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BIOHYDROMETALLURGY

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Joseph Salley, Ron G.L. McCready, Paul L. Wichlacz

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MICROMEDIA

FOREWARD

This book comprises presentations from Biohydrometallurgy 89. The meeting, held at Jackson Hole, Wyoming, U.S.A.,in August 1989, attracted more than 230 participants.

Biohydrometallurgy 89 is the fifth symposium of a series which was began in 1977 and now provides a biannual opportunity for discusion of both fundamental aspects and applications of microorganisms in extraction and recovery of metals, industrial waste treatment, and fossil fuel refining.

The trend continued from the two preceeding meetings (Vancouver and Warwick) to come to grips with the real-world problems arising from both industrial activities and needs, and the regulatory requirements imposed by political entities. Fundamental studies have likewise advanced, commensurate with the demands of developing new technologies. The formal presentations and accompanying discussions reflect both the basic philosophy and the essential details of ongoing work. Poster sessions expanded the broader picture of the level of interest and the current trends in Biohydrometallurgy. It is evident that increasing effort is being devoted to waste treatment and fossil fuel refining studies.

Commercial applications of biohydrometallurgy, while as yet relatively few in number, weigh heavily in importance. Direct economic benefits from modified or alternative extraction processes and waste treatment methods appear substancial in an era of increasing concern for our environment. Further, the economics of biohydrometallurgical and biotechnological processes improve visibly with depletion of our resources and increasing regulation. Whatever the incentive, a strong, worldwide interest in this technology was demonstrated at this meeting.

ACKNOWLEDGEMENT

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Thanks in large measure are due to the many individuals whose dedicated efforts carried the project to successful completion.

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LABORATORY STUDIES/BASE METALS

FACTORS AFFECTING BACTERIAL MINERAL OXIDATION: THE EXAMPLE OF CARBON DIOXIDE IN THE CONTEXT OF BACTERIAL DIVERSITY

P.R. NORRIS

Department of Biological Sciences
University of Warwick
Coventry, England

ABSTRACT

The oxidation of pyrite at 30-68°C during the growth of phylogenetically-diverse, mineral-oxidizing bacteria in air-lift reactors has been assessed in relation to the carbon dioxide concentration of the gas supplied to the vessels. As expected, when the CO_2 concentration did not limit the bacterial activity, the highest rate of mineral oxidation resulted from the activity of thermophiles at temperatures beyond those permitting the growth of *T. ferrooxidans*. The rate of iron solubilization by *Sulfolobus* was reduced during growth in medium gassed with air that was not enriched in CO_2 but was still three to four times more rapid than that by *T. ferrooxidans*. In contrast, the activity of moderate thermophile strain BC1 was greatly reduced to less than that of the mesophile. The protein profiles of the thermophiles grown under different CO_2 concentrations have been examined using electrophoresis to reveal specific proteins that are probably involved in CO_2 fixation and whose concentration relative to the total cell protein reflects the availability of CO_2 to the bacteria. The alterations in the concentrations of these proteins in response to the CO_2 concentration are discussed in relation to the different pathways of CO_2 assimilation in the bacteria.

LES FACTEURS MODIFIANTS L'OXYDATION MINÉRALE BACTÉRIENNE : L'EXEMPLE DU GAZ CARBONIQUE DANS LE CONTEXTE DE LA DIVERSITÉ BACTÉRIENNE

P.R. Morris

Département des Sciences Biologiques
Université de Warwick Coventry, Angleterre U.K.

RÉSUMÉ

L'oxydation de la pyrite dans des bio-réacteurs à ascension pneumatique, à des températures entre 30-68°C durant la croissance de bactéries phylogénétiquement différentes, capables d'oxyder des minéraux, a été vérifiée en fonction de la concentration de gaz carbonique fourni aux récipients. Comme prévu, lorsque la concentration de CO_2 ne limitait pas l'activité bactérienne, l'activité de thermophiles à des températures supérieures à celles qui permettent la croissance de *Thiobacillus ferrooxidans* était responsable du taux maximal d'oxydation. Le taux de solubilisation du fer par *Sulfolbus* a diminué lorsque de l'air non-enrichi en CO_2 était insufflé dans le milieu de culture, il a cependant demeuré de trois à quatre fois plus rapide que celui de *T. ferrooxidans*. Par contre, l'activité de la souche modérément thermophile BC1 a été plus faible que celle de la souche mésophile.

Les profils protéiques des thermophiles après croissance à différentes concentrations de CO_2 ont été analysés par électrophorèse, et ont révélé la présence de protéines spécifiques qui sont probablement impliquées dans la fixation du CO_2, et dont la concentration par rapport à la quantité totale de protéine cellulaire indiquerait la quantité de CO_2 disponible pour la bactérie. Les variations dans les concentrations de ces protéines par rapport à la concentration de CO_2 sont discutées en fonction des différentes voies d'assimilation du CO_2 par les bactéries.

INTRODUCTION

Thiobacillus ferrooxidans or rather poorly characterized, mixed cultures in which this iron- and sulphur-oxidizing mesophile was assumed to be the dominant species, have been used almost exclusively in the development of processes for mineral oxidation by bacteria in reactors. The activity of *T. ferrooxidans* has, therefore, become a standard to which the leaching performance of other potentially useful bacteria can be compared. *Leptospirillum ferrooxidans* and a variety of thermophiles have received most attention in this context (Norris, 1988, 1990) but other bacteria, *Thiobacillus prosperus* for example (Huber and Stetter, 1989), will undoubtedly also attract attention. All of these bacteria share with *T. ferrooxidans* their acidophily and capacity to oxidize iron, but their phylogenetic diversity reflects differences in some aspects of their metabolism which concern growth on mineral sulphides. Attempts to improve the performance of mineral-oxidizing bacteria or to optimize process conditions that influence their activity might relate directly to reactions in mineral dissolution, i.e., iron and sulphur oxidation, or might involve factors such as nutrient assimilation which could affect mineral oxidation indirectly through influencing bacterial growth.

The oxidation of iron ... a direct factor in mineral oxidation

One of the distinguishing features between some major groups of taxonomically-distinct, mineral-oxidizing bacteria is the nature of the cell surface that of *T. ferrooxidans* and *L. ferrooxidans* being Gram negative, that of the most-studied moderate thermophiles being Gram positive (see Karavaiko *et al.*, 1988; Lane *et al.*, 1988), and *Sulfolobus* and *Acidianus* belonging to the archaebacteria. Differences in the composition and structure of the cell envelopes, generally considered to be the site of iron oxidation (Dugan and Lundgren 1965; Ingledew, 1982), likely dictate, therefore, some strain specific features of the interaction with iron. Furthermore, a preliminary examination by optical spectroscopy of the respiratory chains of the bacteria in these groups indicated a variety of components likely to be involved in the subsequent transfer of electrons from ferrous iron (see Norris, 1990). For example, an apparently unique, acid stable cytochrome was revealed in *L. ferrooxidans* and postulated to occupy a position in iron oxidation analogous to that of the periplasmic, acid stable rusticyanin of *T. ferrooxidans* (see Norris, 1989). Some different affinities for ferrous iron and some different tolerances with regard to inhibition of the iron oxidation by ferric iron have been observed among the various mineral-oxidizing bacteria (Norris *et al.*, 1988); iron oxidation by *L. ferrooxidans* in particular being less affected by a decrease in ferrous and increase in ferric iron concentration than that of *T. ferrooxidans*. This factor, together with the apparently greater tolerance of *L. ferrooxidans* of increasing acidity (Norris, 1983; Helle and Onken, 1988), might explain the capacity of *L. ferrooxidans* to compete successfully with *T. ferrooxidans* during growth on pyrite. However, any attempt to relate the observed kinetics of the cell-metal interactions to features of the iron oxidation systems at the molecular level must await the full characterization of the latter. An increased tolerance of ferric iron might be a useful attribute of *T. ferrooxidans* strains intended for application in reactor leaching of some mineral sulphide concentrates, but it is doubtful if a capacity for more rapid iron oxidation, even if it were attainable through strain manipulation or selection, would be generally useful. The rate of biochemical reactions in mineral sulphide oxidation are likely to be limited by availability of substrate from the solid with the rate of mineral dissolution controlled by interacting, limiting factors that would include the particle size, diffusion rates at the mineral surface and the temperature. The bacteria generally grow more rapidly on soluble sulphur compounds and iron than on mineral sulphides. An increase in the temperature, for example, allows more rapid mineral dissolution by thermophilic bacteria growing at temperatures above the limit for *T. ferrooxidans* activity even though the rates of autotrophic growth of the thermophiles on ferrous iron in solution are similar to that of the mesophile (Norris, 1990).

Carbon dioxide ... indirectly affecting mineral oxidation

A factor that indirectly affects mineral oxidation by bacteria and one that may be controlled in reactor mineral processing is the availability of carbon dioxide. As with iron oxidation, the efficiency of carbon dioxide assimilation by various mineral-oxidizing acidophiles might be expected to differ given the aforementioned diversity of the bacteria.

T. ferrooxidans assimilates carbon dioxide principally through the Calvin cycle. One of the key enzymes of this cycle, ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) has been characterized in work with *T. ferrooxidans* that also showed growth on ferrous sulphate was limited unless the concentration of CO_2 in the air gassing the culture was increased (Holuigue *et al.*, 1987). This observation contrasts with those in which the normal concentration of CO_2 in air was sufficient to avoid limiting the growth of *T. ferrooxidans* on ferrous sulphate (Kelly and Jones, 1978) and to avoid limiting mineral sulphide oxidation unless the solids concentration was above a few percent (w/v) (Torma *et al.*, 1972). *T. ferrooxidans* probably responds to CO_2 limitation by increasing the cellular concentration of RuBisCo (Codd and Kuenen, 1987).

The activity of RuBisCo during autotrophic growth of the moderately thermophilic iron-oxidizing bacteria has been demonstrated (Wood and Kelly, 1985). However, with the moderate thermophiles so far studied, autotrophic growth utilizing ferrous iron has been consistently and clearly restricted unless cultures were incubated under air in which the CO_2 concentration was enhanced (Marsh and Norris, 1983a; see Brierley and Brierley, 1986). The capacity of these bacteria to degrade mineral sulphides rapidly in the absence of organic nutrients has been demonstrated with cultures gassed with 1-5% (v/v) CO_2 in air (Marsh and Norris, 1983b; Norris *et al.*, 1986).

In contrast to the eubacteria (*T. ferrooxidans* and moderate thermophile strain BC1), the mineral-oxidizing archaebacteria such as *Sulfolobus* and similar extreme thermophiles do not assimilate CO_2 via the Calvin cycle. The pathway of CO_2 fixation in these bacteria has not been resolved but a reductive carboxylic acid cycle (Kandler and Stetter, 1981) and a possible role for an acetyl CoA carboxylase (Norris *et al.*, 1989) have been suggested. Previous demonstrations of the rapid and efficient oxidation of finely-ground mineral sulphides by *Sulfolobus* in the absence of organic nutrients have generally involved gassing cultures with 0.5 or 1% (v/v) CO_2 in air (Marsh *et al.*, 1983; Norris and Parrott, 1986; Le Roux and Wakerley, 1988).

This paper examines the effect of potentially growth-limiting CO_2 concentrations in air on bacterial growth-associated pyrite oxidation and on the concentration of specific proteins in the thermophilic bacteria. An understanding of the biochemistry of the CO_2 fixation pathways in these bacteria and the responses to CO_2 limitation should benefit any development of a strategy for improving or optimizing mineral oxidation in relation to the assimilation of this essential nutrient.

MATERIALS AND METHODS

Organisms. The growth on minerals of the strains used, *T. ferrooxidans* DSM 583, moderate thermophile strain BC1 and *Sulfolobus* strain BC, has been described before (Marsh and Norris, 1983b; Marsh *et al.*, 1983; Norris *et al.*, 1986; Norris, 1988). In this paper, use of the genus name *Sulfolobus* hereafter refers to *Sulfolobus* strain BC; this isolate is probably a strain of *S. acidocaldarius*, but this has not been confirmed.

Growth on pyrite. Water-jacketed, air-lift reactors (500 ml) with a central draft tube were used. The mineral (40% w/w iron; particles <75 μm diameter) was added to a medium (initially adjusted to pH 2 with H_2SO_4) which contained (g l^{-1}) $(NH_4)_2SO_4$ (0.4), $mgSO_4 \cdot 7H_2O$ (0.5) and K_2HPO_4 (0.2). Air or CO_2-enriched air (250 ml min^{-1}) as indicated in the text was introduced into the reactors at the base of the draught tubes. All inocula (10% v/v) were pyrite-grown. Mineral dissolution was measured as the increase in the iron concentration in solution by atomic absorption spectrophotometry analysis of supernatants from reactor samples after centrifugation.

Polyacrylamide gel electrophoresis (PAGE). Proteins were stained with silver following standard techniques of SDS-PAGE (12% acrylamide gels).

RESULTS

Growth-associated pyrite oxidation under different CO_2 concentrations

In order to consider the activity of the mineral-oxidizing bacteria in relation to the efficiency of their CO_2 assimilation, the increase in the concentration of dissolved iron during their growth (Fig. 1) was taken as a reflection of the pyrite oxidation. However, there was some precipitation of released and oxidized iron, particularly at the highest temperature and during the early phase of the growth of *Sulfolobus* before extensive acidification of the medium. The more gradual increase to the maximum rate of iron solubilization with *Sulfolobus* than with the other bacteria may partly reflect such precipitation. In addition, however, the onset of the maximum rate of iron solubilization by *Sulfolobus* was delayed slightly less than with *T. ferrooxidans* in the absence of enhanced concentrations of CO_2 (for example, Fig. 1). A further comparison of the influence of the CO_2 concentration on mineral oxidation was made in terms of the maximum rates of iron solubilization that were attained (Fig. 2). Culture gassing with air that was not enriched with CO_2 had little effect on the release of iron by *T. ferrooxidans* but allowed only poor growth of the moderate thermophile strain BC1. The rate of iron solubilization by *Sulfolobus* was reduced by an average of 20% at a particular mineral concentration but was still three to four times more rapid than that by *T. ferrooxidans*. The addition of CO_2 to only 0.1% (v/v) in air restored the rates of mineral oxidation by both the moderate and extreme thermophiles almost to those seen at higher CO_2 concentrations (Fig. 2). The influence of the CO_2 concentration is seen here in terms of iron release and therefore on the activity of the bacteria rather than on their growth. During growth of *Sulfolobus* on thiosulphate or tetrathionate in continuous culture under conditions of CO_2 excess (5% v/v CO_2 in air) or limitation (air only), substrate oxidation was uncoupled from growth when CO_2 was limiting (Norris *et al.*, 1989); equivalent oxidation rates were obtained with a concentration of CO_2-limited biomass that approached only half that of cells grown in the presence of excess CO_2. In terms of the growth on pyrite (Fig. 2), microscopy clearly showed that the effect of gassing with air on the numbers of *Sulfolobus* in the cultures was proportionately less than on the rate of iron release when compared to CO_2-sufficient cultures.

Cell protein profiles of bacteria grown under different CO_2 concentrations

Sulfolobus. When *Sulfolobus* was grown under CO_2 limitation in earlier experiments, the concentration of a protein of about 330 kDa was forced to leave considerably increased (Norris *et al.*, 1989). This protein, which carboxlyated acetyl CoA, appeared to have large (59 kDa) and small (19.5 kDa) subunits, with the 59 kDa subunits most noticeable on SDS-polyacrylamide gels of whole-cell proteins. Electrophoresis of solubiliquid protein foam cells taken from reactors during the pyrite oxidation experiment summarized in fig. 2 showed that the concentration of the 59 kDa polypeptide (arrowed, Fig. 3) increased in response to the reduction in the CO_2 concentration. The production of the protein increased, relative to total cell protein, when the CO_2 concentration was reduced from 1 to 0.1% even though the leaching activity was not affected (Fig. 2). It is possible that with 0.1% (v/v) CO_2 in air, some uncoupling of mineral oxidation from growth occurred giving a higher specific rate of oxidation as noted above with growth on soluble sulphur compounds under air. As the concentration of this protein could be increased further, as it was under air (Fig. 3), it is also possible that the bacteria were responding to the intermediate CO_2 concentration by producing only sufficient extra enzyme to increase the CO_2 assimilation efficiency to allow growth equivalent to that obtained when the CO_2 was not limiting. However, it remains to be proven that growth-associated CO_2 fixation is primarily dependent on the activity of this protein. The concentration of the 59 kDa polypeptide at least appears to be a sensitive indicator with regard to the availability of CO_2 to the bacteria. Following the further oxidation of 1% (w/v) pyrite after transfer of cells from growth under air to under CO_2-enriched air, and vice-versa, the 'protein record' was that of the prevailing growth conditions (Fig. 4) but the rate of synthesis or degradation of the indicator protein in response to a changing CO_2 concentration has not been determined.

The moderate thermophile strain BC1. Solubilized proteins from strain BC1 were subjected to SDS-PAGE following growth of the bacteria on pyrite in medium that was supplemented with yeast extract (data not shown) and growth in reactors gassed with different concentrations of CO_2 in air (data of Fig. 2). The enzyme RuBisCo, from whatever source, generally comprises large (usually about 56 kDa) and small (about 15 kDa) subunits, although there are rare exceptions, such as that with only large subunits in some anoxygenic photolithotrophic bacteria. The autotrophically-growing strain BC1 possessed a polypeptide of 56 kDa (upper arrows, Fig. 5) which could be the large subunit of RuBisCo with its concentration appearing to increase in response to the decreasing concentration of CO_2 in the gas supply to the culture. A significant amount of this protein would not have been expected in the bacteria grown in the presence of yeast extract which represses CO_2 fixation during litho-heterotrophic growth (Wood and Kelly, 1983). It is interesting to speculate further on the nature of the polypeptide appearing on the gel just below and apparently about 4 kDa smaller than the putative ruBisCo large subunit. In the autotrophically-growing bacteria and in contrast to the 56 kDa protein, the concentration of this protein appeared to decrease with the reduction in the CO_2 concentration. Further work is required to establish whether this indicates a system analogous in some way to that in the phototrophic *Chromatium* where a slower mobility in polyacrylamide gels of an active form of RuBisCo in comparison with an altered inactive form has been proposed to indicate some post-translational control of the RuBisCo activity (see Tabita, 1988).

A second enzyme unique to the Calvin cycle is phosphoribulokinase (PRK) which catalyses the phosphorylation of ribulose 5-phosphate to give the ribulose 1,5-bisphosphate that is carboxylated by RuBisCo. The PRK of most bacteria comprises subunits of 32-36 kDa. A polypeptide of 35 kDa appeared abundant in thermophile strain BC1 from the reactor gassed with air (lower arrow, Fig. 5). It is possible, but not yet confirmed, that this protein could be PRK that was significantly over-produced only at a level of CO_2 limitation more severe than that which induced the apparent increase in RuBisCo synthesis or activity.

CONCLUSIONS

The CO_2 limitation of the activity of the thermophiles was almost completely alleviated with only 0.1% (v/v) CO_2 in air even though analyses of cell proteins indicated that these bacteria were affected and responding to CO_2 limitation at this concentration. The use of CO_2-enriched air would probably be required to maintain the optimum activity even of *T. ferrooxidans* where tested at higher mineral concentrations (Torma *et al.*, 1972) than those used in this work but which would be desirable for an industrial process. It seems likely, but remains to be shown, that adequate growth of the moderate thermophile under such conditions would require a higher concentration of CO_2 than either *T. ferrooxidans* or *Sulfolobus*. In reactors gassed with air, the activity of Sulfolobus was still far greater than that of *T. ferrooxidans*, even without the CO_2 concentration it required for maximum activity. However, the inhibition of *Sulfolobus* by agitation in the presence of high concentrations of minerals (Norris and Barr, 1988) still restricts its potential industrial application.

The visualization of the regulated increase in the concentrations of specific proteins in the thermophiles in response to CO_2 limitation could provide a useful means of assessing the status of the bacteria in reactors in relation to the CO_2 availability. The absence of any over-production of the key proteins would illustrate that excess CO_2 was present. In practice, however, with mixed rather than pure cultures, the picture would likely be obscured, at least with the moderate thermophiles by the presence of Gram negative, sulfur-oxidizing bacteria, (see Norris, 1990) which possess RuBisCo and fix CO_2 from air efficiently.

An explanation of the poor efficiency of assimilation of CO_2 from low concentrations in air by the Gram positive, moderately thermophilic, iron-oxidizing bacteria may be sought by comparing the concentrations, activities and characteristics of the key enzymes of *T. ferrooxidans*. Possible differences in the process of CO_2 uptake and, therefore, in its concentration, directly available to the RuBisCO in the different bacteria should also be examined. The identification of a rate-limiting step in CO_2 assimilation by the moderate thermophiles could provide a target for strain improvement; this could parallel attempts to select or isolate strains or new species which might prove more cost effective with respect to the CO_2 provision for mineral processing at 40-55°C.

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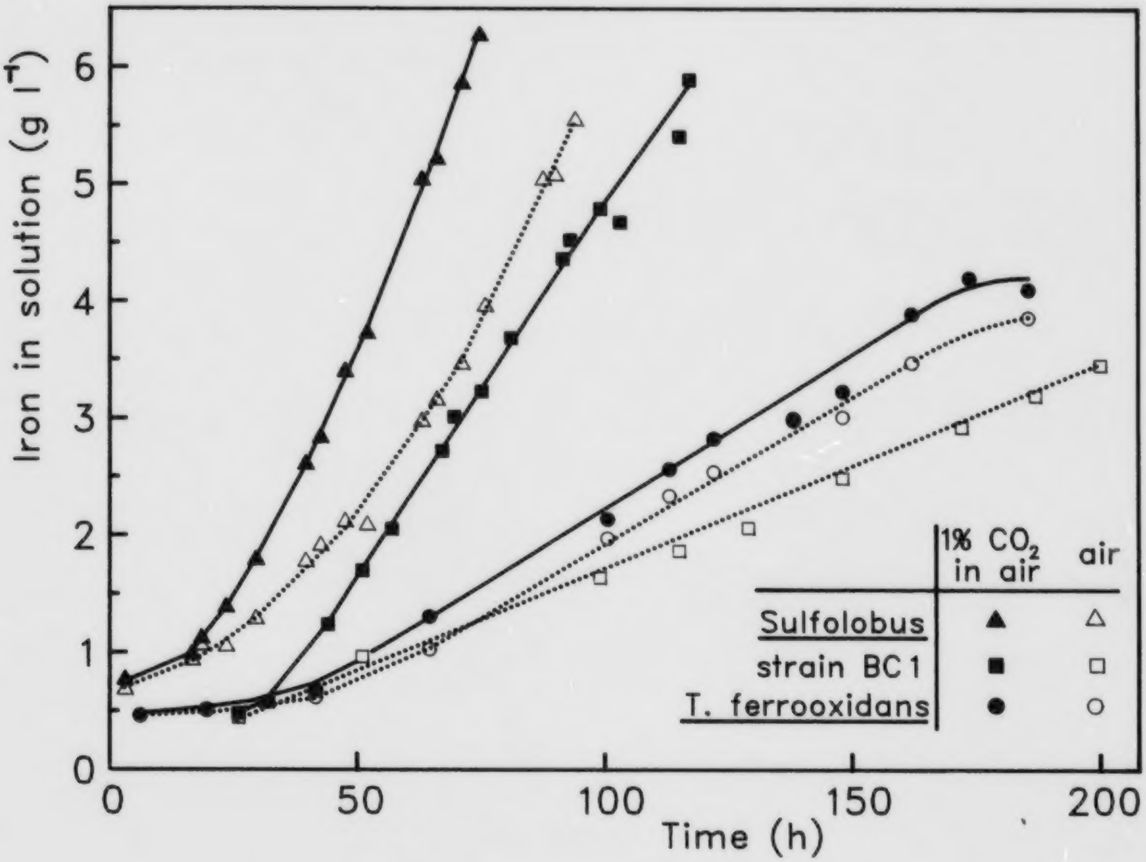

Fig. 1 Iron solubilization from pyrite (2% w/v) during growth of *T. ferrooxidans*, moderate thermophile strain BC1 and *Sulfolobus* at 30, 48 and 68°C respectively in reactors gassed with air or CO_2-enriched air as indicated.

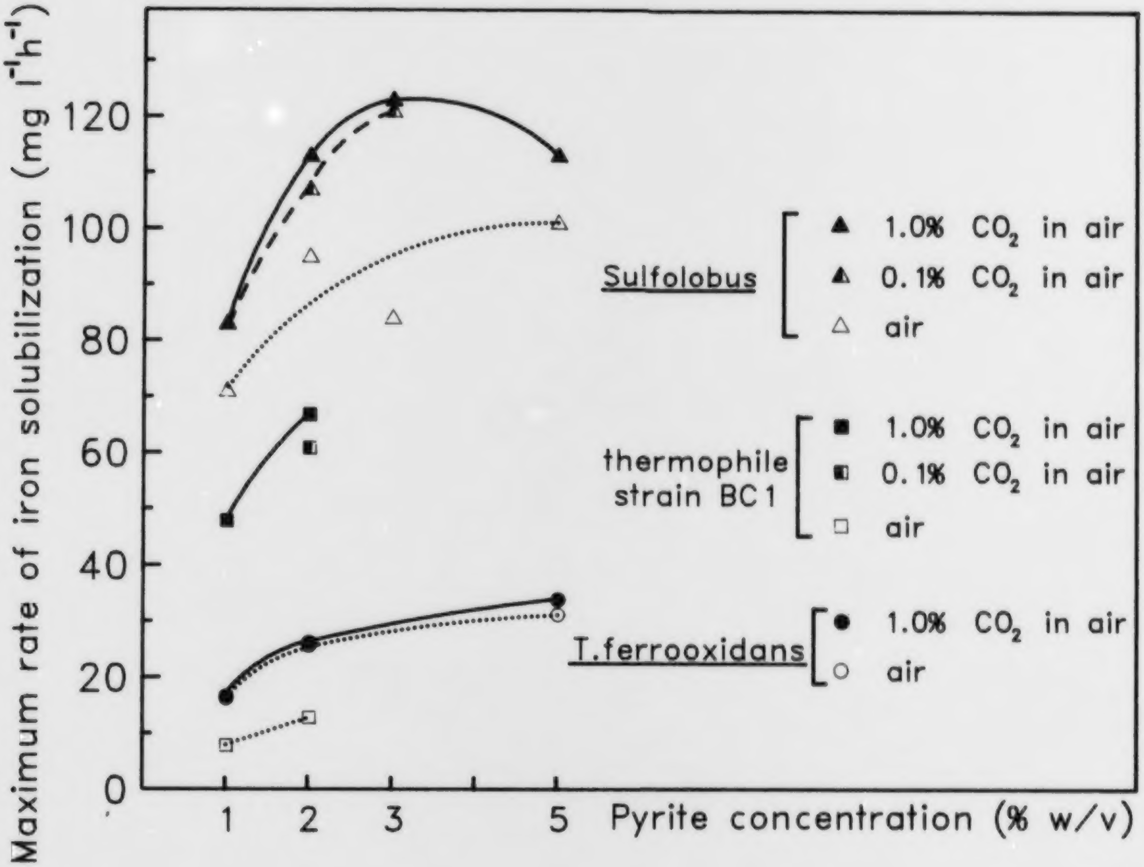

Fig. 2 The maximum rates of iron solubilization from pyrite using mesophilic and thermophilic bacteria and the indicated CO_2 concentrations in the reactor gassing (see Fig. 1 for the growth temperatures and examples of the leaching curves).

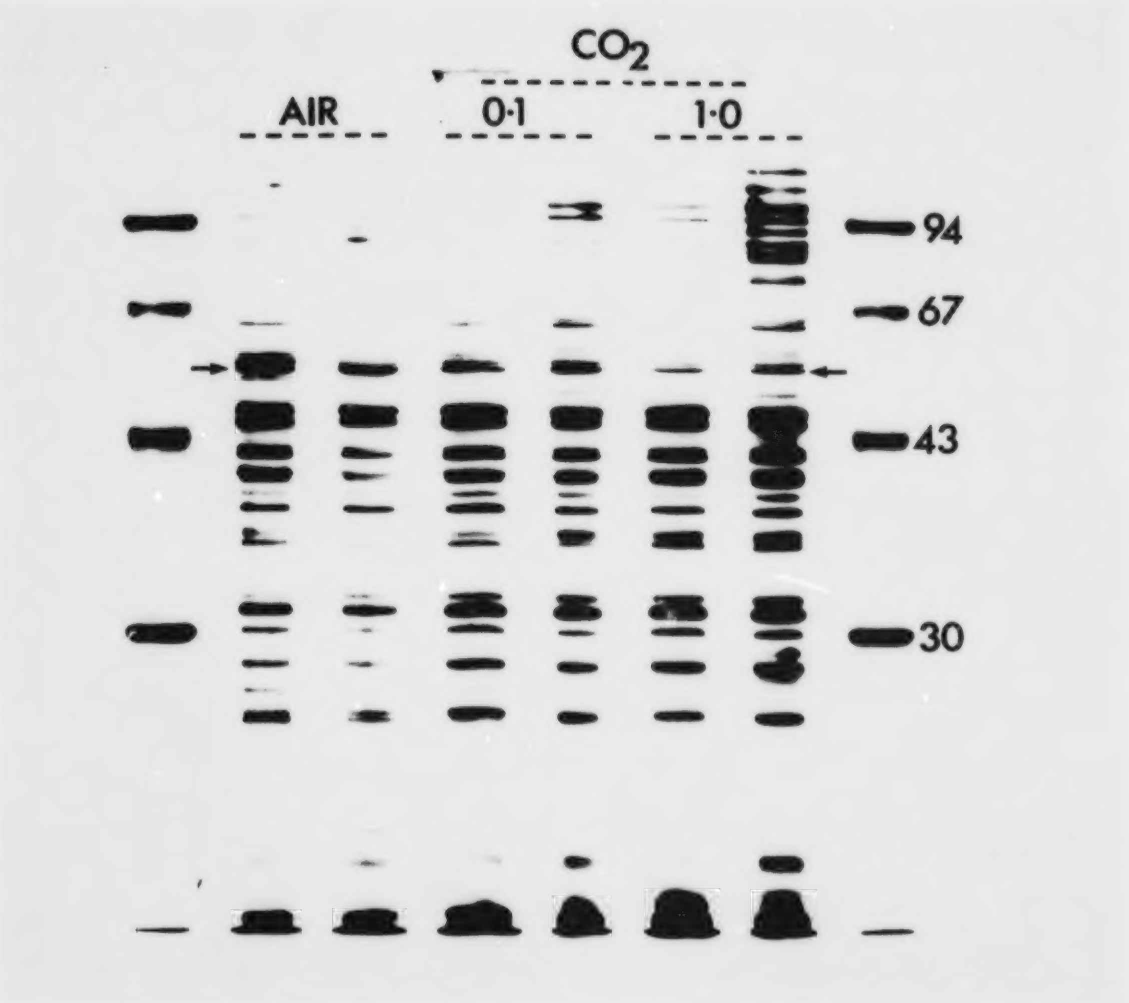

Fig. 3 Protein SDS-PAGE of *Sulfolobus* from reactors gassed with air, 0.1 or 1% (v/v) CO_2 in air as indicated. Solubilized proteins of bacteria from reactors containing 2% and 3% (w/v) pyrite (leaching rates on Fig. 2) were run in the lefthand and righthand tracks respectively of the pairs under each gassing mixture heading. The protein standard markers are in kDa and the 59 kDa protein discussed in the text is indicated by an arrow.

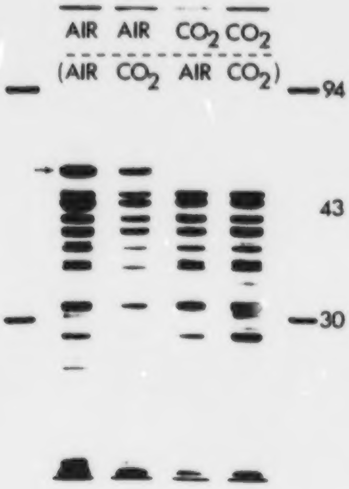

Fig. 4 Protein SDS-PAGE of *Sulfolobus* grown in four reactors which initially contained 1% (w/v) pyrite. Each reactor was gassed with air or 5% (v/v) CO_2 in air (upper line of key) and inoculated with bacteria grown under air or 5% (v/v) CO_2 in air (bracketed, lower line of key). The 59 kDa protein is indicated by an arrow. Markers are in kDa.

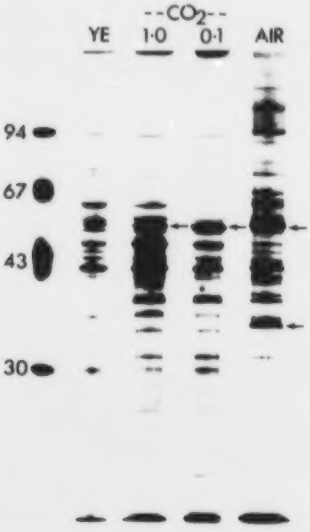

Fig. 5 Protein SDS-PAGE of moderate thermophile strain BC1. the bacteria were grown on 2% (w/v) pyrite in reactors gassed (as indicated on the figure) with air, 0.1 or 1% (v/v) CO_2 in air (leaching rates on Fig. 2) or with air through medium also supplemented with yeast extract (YE, 0.02% w/v). The putative RuBisCo (upper arrows) and PRK (lower arrow) are indicated (see test). The protein standard markers are in kDa.

BIOLEACHING OF VALUABLE METALS FROM SILICATE ORES AND SILICATE WASTE PRODUCTS

K. Bosecker,

Federal Institute for Geosciences and Natural Resources (BGR), Stilleweg 2,
D-3000 Hannover 51, Federal Republic of Germany

ABSTRACT

Heterotrophic microorganisms isolated from various mine sites in Australia, Portugal and Romania and screened for acid production were investigated for their use in dissolving valuable metals (Al, Ni, Cr, Fe, Ti) from silicate ores and from waste products from the aluminium processing industry. All strains of bacteria were found to be sensitive to metal concentrations > 100 ppm, and none of them was capable of dissolving silicon. Among fungi, *Penicillium* pre-dominated. Strains of *P. simplicissimum* were most abundant and most suitable for leaching purposes because they were not affected by metal concentrations in the range of 1,000 — 1,500 ppm. After adaptation, some strains still grew at 10,000 ppm Ni^{2++}, 6,000 ppm Cr^{6++} or 6,000 ppm Al^{3++}.

With citric acid, 77% of the nickel and 31 % of the aluminum was extracted from a silicate-type of ore and 60% of aluminium was recovered from industrial waste. After thermal pre-treatment at 650°C, 90% of nickel and 60% of aluminium in the ore was solubilized. Dissolution of chromium and titanium was comparatively poor.

BIOLIXIVIATION DE MÉTAUX AYANT UNE VALEUR COMMERCIALE DANS DU MINERAI ET DES RÉSIDUS SILICEUX

K. Bosecker

Institut Fédéral des Géoscience et des Ressources Naturelles (BGR),
Hanovre, République Fédérale d'Allemagne

RÉSUMÉ

Des micro-organismes isolés sur différents sites miniers en Australie, au Portugal et en Roumanie, ayant été sélectionnés en fonction de leur aptitude à produire des acides, ont fait l'objet d'essais visant à déterminer leur capacité de dissoudre des métaux (Al, Ni, Cr, Fe, Ti) en concentration suffisante dans des minerais siliceux et des résidus siliceux rejetés par les alumineries. Au cours des essais, toutes les souches de bactéries ont été sensibles à des concentrations de métaux supérieures à 100 ppm et aucune n'a été capable de dissoudre le silicium. Parmi les moisissures, *Penicillium* était prédominant. Les souches de *P. simplicissimum* étaient les plus abondantes et les mieux adaptées à la lixiviation parce qu'elles n'étaient pas sensibles à des concentrations de métaux de l'ordre de 1 000 à 1 500 ppm. Après adaptation, certaines souches ont continué de croître en présence de concentrations de Ni^{2+} de 10 000 ppm, de Cr^{6+} de 6 000 ppm ou de Al^{3+} de 6 000 ppm.

En présence d'acide citrique, 77 pour cent du nickel et 31 pour cent de l'aluminium ont été extraits du minerai siliceux tandis que 60 pour cent de l'aluminium a été récupéré de résidus industriels. Après pré-traitement thermique (650°C), 90 pour cent du nickel et 60 pour cent de l'aluminium présents dans le minerai ont été mis en solution. Par comparaison, la dissolution du chrome et du titane à été faible.

INTRODUCTION

Heterotrophic microorganisms are known to contribute to the release of heavy metals from rocks and minerals. In contrast to the chemolithoautotrophic thiobacilli which are already used commercially for the leaching of sulfide ores, the heterotrophic microorganisms require organic carbon sources for their growth and energy supply and do not have any benefit from the degradation of minerals. Heterotrophic leaching is due to the production of organic acids and other metabolic compounds which are excreted into the environment and dissolve heavy metals by direct displacement of metal ions from the ore matrix by hydrogen ions and by the formation of soluble metal complexes and chelates (Duff *et al.*, 1963; Henderson and Duff, 1963; Wagner and Schwartz, 1967; Berthelin, 1977; Eckhardt, 1979).

These mechanisms have a potential for the recovery of valuable metals from non-sulfide minerals which cannot be treated by the conventional autotrophic bacteria and offer possibilities for developing a new biotechnology process engineering for covering our future demand for metalic raw materials. Meanwhile special importance is attached to heterotrophic leaching for the recovery and enrichment of precious metals such as gold (Karavaiko *et al.*, 1977, pp. 61 - 64) and rare earth elements (Glombitza *et al.*, 1988).

As previously shown (Bosecker, 1986; McKenzie *et al.*, 1987), heterotrophic microorganisms can be used for the dissolution of nickel from laterite nickel ores. Up to 72% of the nickel was extracted with citric acid produced by the filamentous fungus *Penicillium*. In addition to nickel, laterite nickel ores often contain significant amounts of other valuable metals such as aluminum and chromium whose extraction has been neglected so far. Therefore one of the objectives of our research was to look for the leachability of these metals.

In all industrial countries huge amounts of hazardous waste products are accumulated, the deposition and treatment of which cause environmental and economic problems. Very often the mineral waste products contain high amounts of valuable metals. Controlled dissolution of these metals may contribute to a future guarantee of raw material supply and simultaneously to a decontamination of mineral waste products. It will reduce environmental problems and deposition expenses. Residues from the aluminum producing industry for example, still contain considerable concentrations of aluminum and sometimes titanium as well. Solubilization of aluminum by metabolites of *Aspergillus niger* and *Penicillium simplicissimum* has been indicated by several authors (Silvermann and Munoz, 1970; Mehta *et al.*, 1979; Groudev *et al.*, 1982) but to our knowledge nothing has been reported on the leaching of aluminum from residues of aluminium production. As the German aluminum processing industry entirely depends on the import of bauxite materials, recycling of aluminum from waste products would be most beneficial. Therefore microbial leaching of aluminum from silicate waste products became the other objective of our investigations.

MATERIALS AND METHODS

Microorganisms

Heterotrophic microorganisms suitable for the solubilization of nickel from silicate ores were isolated from rock and soil samples from the laterite nickel deposit at Greenvale, Australia (Bosecker, 1986). The same procedure was used for isolating new strains of heterotrophic bacteria and fungi from rock and water samples collected in a test gallery of a slightly alkaline, nickel-containing serpentinite massif and at mining dumps of former mines of silicate manganese ore deposits in Romania. Additional strains were isolated from mine water in a copper mining area in Portugal. All isolates were screened for acid production by spreading single colonies on solid media containing phenol red or methyl red and bromthymol blue as pH indicators or by pH measurements in the liquid medium.

Screening for "silicate" bacteria was carried out using the Bogdanovic medium as described by Groudev *et al.*, (1982).

All isolates were tested for their sensitivity to nickel, aluminum, ferric iron, chromium (III) and chromium (VI) by gradually increasing the metal ion concentration in the culture medium.

Leaching Materials

Laterite nickel ores were obtained from major deposits in Brazil and from the Dominican Republic. The latter was kindly supplied by Falconbridge Ltd., Canada. Nickel-containing serpentinite was received from the Romanian Ministry of Mines. Residues of aluminum-producing processes (red mud, incineration cinders, ashes) were obtained from the Germany aluminum industry (VAM).

Leaching Technique

Leaching tests were carried out as shake-flask experiments. Experimental details including analytical methods were reported earlier (Bosecker, 1986).

RESULTS AND DISCUSSION

Isolation and Characterization of Heterotrophic Bacteria

Altogether 34 strains of heterotrophic bacteria were isolated from rock and water samples. Six strains showed continuous acid production whereas eight isolates caused a decrease in pH only during the initial growth phase. Twenty-one strains grew on silicate-containing media, six of which had been identified as acid producers. So far, these strains have not shown any evidence of silicon solubilization when grown in the presence of silicate ores.

All bacteria-strains were found to be sensitive to metal concentrations > 100 ppm. Adaptation to higher concentrations was extremely problematic and was stopped for this reason.

Characterization of Isolated Fungi

A total 36 strains of fungi were isolated from mine water and rock samples; 28 of them were identified as acid-producing strains and investigated for their use of leaching purposes. Most acid-producing strains were found to belong to the genus *Penicillium* (12). Eight filamentous fungi have not yet been identified, and six other acid-producing isolates have been classified as yeasts.

The filamentous fungi were found to be less sensitive to heavy metals than the yeasts. The former were adapted to 1,000 ppm of nickel, aluminum, iron or chromium without any difficulty whereas the yeast isolates did not tolerate metal concentrations > 300 ppm. Strains of *P. simplicissimum* were not affected by metal concentrations in the range of 1,000 - 1,500 ppm. After adaptation, some strains still grew at 10,000 ppm Ni^{2+}, 6,000 ppm Cr^{6+} or 6,000 ppm Al^{3+}. Some of the most striking results are summarized in Table 1. Although nickel has been reported to be one of the most toxic elements for *Aspergillus niger* (Avakyan, 1974), two strains obtained from the German Type Culture Collection (DSM) were adapted to 1,000 ppm Ni^{2+}. Strains of *A. fumigatus* isolated from rock samples were found to be active at 2,000 - 3,000 ppm Ni^{2+}.

Leaching with heterotrophic microorganisms

As far as silicate leaching with heterotrophic bacteria is concerned, our former results were confirmed. The new isolates although found in silicate environments and specially selected for acid production and growth on silicate-containing media did not dissolve noticeable amounts of nickel or aluminum from silicate ores. So far, we have not observed silicon extraction as reported by Groudev and Groudeva (1986). For that reason, leaching with heterotrophic bacteria was discontinued.

Among 63 fungi kept in our stock collection, 28 strains selected on account of strong acid production and high tolerance of the metals to be extracted were investigated for their leaching effectiveness using a laterite nickel ore from Brazil. This ore was chosen because most earlier leaching tests had been carried out with this ore. Only a few results are summarized in Table 2 which demonstrate a variety of differences with regard to the strains which were used and the metal which was dissolved. *Penicillium* was the most effective fungus, and nickel was the metal which was leached best. With the exception of strain no. 49 which was kindly supplied by I. Lazar (Bucharest), the new isolates of *Penicillium* strains were less effective than our strain *P. simplicissimum P 6*. Besides nickel, a little aluminum, some iron, different amounts of magnesium, but no chromium were dissolved. The different results when leaching the same ore were be due to the type of acid which was produced and need further investigations.

Leaching of Romanian serpentinite

Within the framework of a German-Romanian cooperation, the possibility of processing nickel containing serpentinite by bio-hydrometallurgical methods was investigated. Because of the good results with our strain *P 6* presented in Table 2, microbial leaching experiments were performed with this strain instead of the indigenous strains. Nevertheless solubilization was very poor and except for aluminum, almost in the range of the sterile control.

To get some information about the leachability of the serpentinite, chemical leaching with various types of organic acids was carried out (Table 3). Surprisingly glycolic acid instead of citric acid was the most effective organic acid for leaching nickel. In the case of aluminum, citric acid was the most effective one. As recommended by Groudev (pers. commun., 1988) thermal pre-treatment increased the leaching of nickel. The best results were achieved when the pretreated serpentinite was leached at about 900°C with citric acid at strongly acidic conditions. Under these conditions about 75% nickel was dissolved. Extraction of aluminum was not improved by thermal pre-treatment, on the contrary, aluminum extraction was less than from the untreated serpentinite. Strongly acidic conditions and leaching at 900°C favored the extraction of aluminum, 19 % aluminum were solubilized within 6 hours. However, due to the low metal content of the mineral, metal concentrations in the liquid were very low. It is concluded that nickel extraction from the serpentinite by microbiological acid production is practically feasible, but there is some doubt whether such a process is economically attractive.

Leaching of silicate laterite nickel ore

Some evidence exists that bioleaching could become an alternative technology for processing the silicate laterite nickel ore from the Dominican Republic (Falconbridge). In contrast to the serpentine rock from Romania, citric acid was the most effective leaching agent although serpentine is the main mineral component in the laterite nickel ore. About 80% of the nickel and 30% of the aluminum were extracted (Table 4). Thermal pre-treatment of the ore, higher temperature during leaching, and acidification to pH 0.5 intensified the leaching process so that within 6 hours, 90% of the nickel and 70% of the aluminum were extracted. Solubilization of iron and magnesium did not change noticeably.

Leaching of silicate waste products

Microbiological leaching of aluminium from silicate ores is well known. Based on this phenomenon a flow sheet for aluminum leaching from mineral raw materials has been developed (Groudev *et al.*, 1983; Groudev, 1988). We investigated microbial leaching of aluminum from silicate waste products which contained considerable amounts of aluminum and other valuable metals. Preliminary experiments to date have been oriented towards a chemical leaching with synthetic organic acids. The first results are presented in Table 5. In the case of ashes and cinders, citric acid was the most effective leaching agent. Up to 60% of aluminum were extracted from incineration cinders. After leaching, the liquid contained about 3g Al/L. With red mud, oxalic acid solubilized 40% of aluminum corresponding to about 5.5 g Al/L in solution. Besides aluminum, a little titanium was solubilized. With the exception of ashes, solubilization of iron ran parallel to the solubilization of aluminum, in contrast to magnesium.

Conclusions

Based on the experimental data, bioleaching of nickel and aluminum from silicate ores and waste products is supposed to be practicable and create new opportunities for the mineral processing industry. However, at present there is some doubt whether such a process will be economically feasible as long as refined sugars are used as substrates for the microbial production of leaching agents. Promising results have been obtained by substituting organic waste products such as sewage from starch and antibiotics production. However, additional intensive studies are needed before heterotrophic leaching can be applied commercially.

Acknowledgements

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Table 1

Metal tolerance of fungi after adaptation

Strain	Species	Source	Metal-concentration (ppm)				
No.			Ni^{2+}	Fe^{3+}	Cr^{3+}	Cr^{6+}	Al^{3+}
1	*Penicillium funiculosum*	Greenvale, Australia	2500	1000	100	1000	3000
2	*Penicillium simplicissimum*	"	15000	1000	4500	1000	6000
3	*Penicillium simplicissimum*	"	5000	4000	4100	13000	7000
6	*Aspergillus fumigatus*	"	3000	600	4000	1500	5000
23	*Penicillium simplicissimum* (P6)		30000	3000	1000	6000	6000
24	*Fusarium oxysporum*	Romania	11000	500	—	6000	4000
25	*Penicillium simplicissimum*	"	10000	1500	3000	6000	3000
29	*Penicillium funiculosum*	"	1500		600	1000	
30	*Aspergillus fumigatus*	"	2000	1000	1000	400	
42	*Penicillium sp.*	Portugal	4000	1000	1000	600	1000
61	*Penicillium sp.*	Romania	1000	1000	1000	1000	1000

Table 2

Leaching of silicate Ni-Ore 424 with fungi isolated from Romanian Mine Sites

Shake flask experiments; Pulp density: 5 g/100 ml

Culture Medium: Duff (8% glucose)

Chemical analysis: 424 (Brazil) Ni = 2,37% Al = 2,09% Cr = 0,77% Fe = 16,35% Mg = 8,33%

No.	Strain Type	t (days)	pH	Ni %	Al %	Cr %	Fe %	Mg %
24	Fusarium	28	6,3 - 4,3 - 7,0	6,9	0,3	0,0	0,0	2,8
25	P. simpl.	28	6,3 - 3,6 - 7,0	26,5	7.0	0,0	5,1	26,2
26	P. simpl.	20	6,5 - 4,2 - 5,3	15,3	3,0	0,4	2,0	8,1
29	P. funic.	23	6,1 - 3,4 - 3,8	18,0	2,7	0,7	3,1	13,5
30	A. fumig.	13	6,0 - 4,4	24,5	2,9	0,1	4,7	17,8
40	Yeast	31	3,9 - 6,3	5,1	0,1	0,0	0,1	4,3
49	P. spec.	28	4,9 - 3,9 - 5,1	41,1	2,6	0,3	2,9	4,7
50	P. spec.	11	4,7 - 2,9 - 3,5	36,1	2,5	0,3	2,9	9,0
23	P. simpl.	29	5,6 - 3,4	48,0	n.d.	n.d.	15,7	50,0
	P 6 sterile	21	6,3 - 6,7	2,1	0,0	0,0	0,0	2,1

Table 3

Chemical leaching of serpentinite (Romania)

Pulp density: 10 g/100 ml

Chemical analysis: 471 Ni - 0,23 % Al = 0,61 % Cr = 0,44 % Fe = 6,54 % Mg = 23,41 %

Treatment	t	pH	Ni		Al		Cr		Fe		Mg	
			ppm	%	ppm	%	ppm	%	ppm	%	ppm	%
Citric acid 0,5 m; pH 1,2	19 d	2,6-4,8	35	15,3	67	11,1	2,5	0,6	1430	21,9	1636	7,0
Oxalic acid 0,5 M; pH 0,9	19 d	0,9-4,3	22	9,5	6	1,1	0,6	0,1	1208	18,5	1109	4,7
Glycolic acid 0,5 M; pH 2,1	19 d	2,7-5,0	100	43,9	0,4	<0,1	0,3	<0,1	620	9,5	2929	12,5
Gluconic acid 0,5 M; pH 2,0	19 d	2,6-4,8	72	31,4	3	0,4	0,5	0,1	426	6,5	2652	11,3
12 h/650°C + Citric acid 0,5 M; pH 1,2	19d	2,9-6,0	147	64,2	4	0,7	5	1,2	770	11,8	1880	8,0
12 h/650° + Citric acid 0,5 M + HCl → pH 0,5 + 6 h/90°C	6 h	0,6-4,2	170	74,6	115	19,0	5	1,2	1694	25,9	2924	12,5

Table 4

Chemical leaching of silicate laterite nickel ore (Dominican Republic)

Shake flask experiments; Pulp density: 10 g/100 ml

Chemical analysis: 462 Ni - 1,63 % Al = 1,06 % Cr = 0,69 % Fe = 14,32 % Mg = 12,77 %

Treatment	t	pH	Ni		Al		Cr		Fe		Mg	
			ppm	%	ppm	%	ppm	%	ppm	%	ppm	%
Citric acid 0,5 m; pH 1,2	19 d	1,2-3,6	1264	77,5	332	31,4	44	6,5	3700	25,9	1092	8,6
Oxalic acid 0,5 M; pH 0,9	19 d	0,9-2,8	134	8,1	56	5,3	24	3,5	4158	28,9	2557	20,0
Glycolic acid 0,5 M; pH 2,1	19 d	2,1-3,9	641	39,2	26	2,4	5	0,7	759	5,3	3328	26,0
Gluconic acid 0,5 M; pH 2,0	19 d	2,0-3,8	577	35,4	74	7,0	4	0,7	1179	8,2	2793	21,9
12 h/650°C + Citric acid 0,5 M; pH 1,2	19d	1,2-3,6	1500	92,0	474	44,8	56	8,1	4040	28,2	1093	8,6
12 h/650°C + Citric acid 0,5 M +HCl → pH 0,5 + 6 h/90°C	6 h	0,5-3,0	1477	90,6	737	70,0	55	8,0	3954	27,6	1342	10,5

Table 5

Chemical leaching of silicate waste products

Shake flask experiments; Pulp density: 10 g/100 ml

Chemical analysis:	412 (red mud)	463 (incineration cinders)	464 (ashes)
Ni	= 0,004 %	0,009 %	0,007 %
Al	= 13,46 %	4,99 %	11,68 %
Fe	= 20,52 %	7,48 %	5,34 %
Mg	= 0,05 %	1,23 %	0,81 %
Ti	= 5,99 %	0,44 %	0,50 %

Treatment	t	pH	Al		Fe		Mg		Ti	
			ppm	%	ppm	%	ppm	%	ppm	%
412										
Citric acid 0,5 m; pH 1,2	20	1,2-2,6	2461	18,3	316	1,5	1,7	3,3	212	3,5
Oxalic acid 0,5 M; pH 0,9	20	0,9-1,8	5476	40,7	4885	23,8	12,5	25,0		
Glycolic acid 0,5 M; pH 2,1	20	2,1-4,2	1221	9,1	134	0,7	34,3	68,5	n.d.	
Gluconic acid 0,5 M; pH 2,0	20	2,0-3,7	1039	7,7	155	0,7	44,2	88,5		
463										
Citric acid 0,5 M; pH 1,2	19	1,2-2,5	2965	59,3	3685	49,3	105	8,5	2	0,4
Oxalic acid 0,5 M; pH 0,9	19	0,9-2,8	2103	42,2	2092	28,0	530	43,1	n.d.	
Glycolic acid 0,5 M; pH 2,1	19	2,1-4,4	1111	22,3	845	11,3	495	40,2		
Gluconic acid 0,5 M; pH 2,0	19	2,0-3,3	997	20,0	1322	17,7	457	37,1		
464										
Citric acid 0,5 M; pH 1,2	19	1,2-2,1	2613	22,4	486	9,1	51	6,2	111	22,4
Oxalic acid 0,5 M; pH 0,9	19	0,9-1,2	1489	12,7	1443	27,0	256	32,5		
Glycolic acid 0,5 M; pH 2,1	19	2,1-3,6	1250	10,7	760	14,2	260	31,9	n.d.	
Gluconic acid 0,5 M; pH 2,0	19	2,0-3,2	622	5,3	418	7,8	423	52,0		

n.d. = not determined

BACTERIAL LEACHING OF CHALCOPYRITE — CONTAINING ORES IN THE PRESENCE OF SILVER AND GRAPHITE IN SHAKE FLASKS

Lasse Ahonen[1§] *and Olli H. Tuovinen*[2*]

[1]*Department of Microbiology, University of Helsinki,*
SF-00710 Helsinki, Finland; and [2]*Department of Microbiology,*
The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210-1292, U.S.A.

ABSTRACT

Bacterial leaching experiments were performed in shake flasks with two copper (chalcopyrite) ore samples. One sample contained pyrite as the predominant iron sulfide and was free of pyrrhotite. The other sample contained pyrrhotite as the predominant iron sulfide and a minor amount of pyrite. The particle size and pulp density of the two ore samples were standardized in these experiments. Copper was dissolved faster from the pyritic sample, suggesting a galvanic effect on chalcopyrite leaching. Silver in trace amounts accelerated the leaching of copper. The effect was proportional to the amount of silver added and was more enhanced with the pyrrhotitic ore sample. Finely ground graphite had also a positive effect on chalcopyrite leaching and was more pronounced with the pyritic sample.

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§ Present address: Geological Survey of Finland, SF-02150 Espoo, Finland.

* Corresponding author.

LA LIXIVIATION BACTÉRIENNE DE MINERAIS CONTENANT DE LA CHALCOPYRITE EN PRÉSENCE D'ARGENT ET DE GRAPHITE DANS DES CONTENANTS AGITÉS

Lasse Ahonen[1§] et Olli H. Tuovinen[2]*
[1] *Département de Microbiologie, Université de Helsinki, SF-00710 Helsinki, Finlande; et*
[2] *Département de Microbiologie, Université de l'État de l;Ohio, 484 ouest, 12ième avenue,*
Colombus, Ohio 43210-1292, E.U.

RÉSUMÉ

Des expériences sur la lixiviation bactérienne ont été exécutées dans des contenants agités avec deux échantillons de minerai de cuivre (chalcopyrite). Un échantillon contenait de la pyrite comme source majeure de sulfure de fer et était exempt de pyrrhotite. L'autre échantillon contenait une faible quantité de pyrite et de la pyrrhotite comme source principale de sulfure de fer. La taille des particules et leur densité de pulpe des deux échantillons de minerai ont été standardisées dans ces expériences. Le cuivre de l'échantillon contenant le plus de pyrite s'est dissout plus rapidement que celui contenant le plus de pyrrhotite, ce qui suggère qu'un effet galvanique est impliqué dans la lixiviation de la chalcopyrite. L'argent à l'état de trace a accéléré la lixiviation de cuivre. L'effet était proportionnel à la quantité d'argent ajouté et était plus considérable avec l'échantillon de minerai contenant la pyrrhotite. De fines particules de graphite ont aussi eu un effet positif sur la lixiviation de la chalcopyrite, cet effet était plus prononcé chez l'échantillon contenant beaucoup de pyrite.

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§ Adresse actuelle : Commission Géologique de la Finlande, SF- 02150 Espoo, Finlande.

* Auteur à qui toute correspondance doit être adressée.

INTRODUCTION

The microbiological leaching of chalcopyrite is a relatively slow reaction by comparison with that of secondary sulfides. Complete bacterial oxidation of the sulfur entity in chalcopyrite yields sulfate (eqn. 1) but partial oxidation which yields elemental sulfur (eqn. 2) is very characteristic of this mineral.

$$2CuFeS^2 + 8.50^2 + 2H^+ \rightarrow 2Cu^2 + 2Fe^{3+} + 4SO_4^{2-} + H_2O \qquad [1]$$
$$CuFeS_2 + 4Fe^{3+} \rightarrow Cu^{2+} + 5Fe^{2+} + 2S \qquad [2]$$

Incomplete oxidation results in the formation of a sulfur layer on chalcopyrite surfaces which hinders the interfacial flux of reactants and products. This is well recognized problem both in the chemical and microbiological leaching of chalcopyrite.

Pyrite has been demonstrated to have a positive effect on chalcopyrite leaching (Mehta and Murr, 1982; Ahonen *et al.*, 1986). Pyrite and chalcopyrite establish a galvanic couple which enhances the anodic dissolution of chalcopyrite. Chalcopyrite oxidation may eventually become diffusion-limited due to the formation of sulfur coating. Because of poor conductivity, sulfur formation also prevents electron transport processes on mineral surfaces.

Pyrrhotite lacks a positive catalytic effect on chalcopyrite leaching, possibly due to the pyrrhotite rest potential which is too low for its cathodic behavior in galvanic coupling with chalcopyrite. An additional feature with pyrrhotite is that elemental sulfur is produced during its microbiological leaching (Ahonen *et al.*, 1986). Elemental sulfur does not usually accumulate during the microbiological leaching of pyrrhotite.

The addition of a silver salt displays an accelerating effect on the chemical (Miller and Portillo, 1981) and microbiological (Ahonen and Tuovinen, 1990 a,b) leaching of chalcopyrite, but the exact mechanism has not been unequivocally elucidated. Silver effect may be based on the transient formation of Ag_2S (eqn. 3) which is oxidized to Ag^+ and S^o by excess Fe^{3+} (eqn. 4).

$$CuFeS_2 + 4Ag^+ \rightarrow 2Ag_2S + Cu^{2+} + 2Fe^{3+} \qquad [3]$$
$$Ag_2S + 2Fe^{3+} \rightarrow 2Ag^+ + 2Fe^{2+} + S^o \qquad [4]$$

Silver ion thus released will again react with sulfur on chalcopyrite surface. The elemental sulfur layer produced upon Ag_2S oxidation is less tenacious, causes less diffusional resistance, and has a higher porosity than the elemental sulfur layer formed without the intermediate silver sulfide. Precipitates may also include elemental silver and silver sulfate on mineral surfaces. These insoluble products are not desired in the leaching process because they are contributing to the loss of catalytic silver. Silver is also incorporated into Fe(III) precipitates such as argentojarosite $[AgFe_3(SO_4)_2(OH)_6]$ in Ag-catalyzed biological leaching of chalcopyrite (Ahonen and Tuovinen, 1990b).

The silver effect is amplified in the presence of iron-oxidizing bacteria such as *Thiobacillus ferrooxidans*. By oxidizing ferrous iron (eqn. 5), these bacteria maintain a favorable ratio of Fe^{3+}/Fe^{2+} and thus contribute to the ferric-iron dependent oxidation of silver sulfide.

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \qquad [5]$$

In the absence of the microbiological oxidation of ferrous iron, silver amendment has a limited effect because the rate of the chemical oxidation at low pH values is negligible and cannot therefore maintain a favorable ration of Fe^{3+}/Fe^{2+}.

The rate of the chemical oxidation of elemental sulfur is insignificant under these conditions and therefore the role of thiobacilli is important also in sulfur oxidation (eqn. 6).

$$2S + 60_2 + 2H_2O \rightarrow 2H^+ + 2SO_4^{2-} \qquad [6]$$

The positive catalytic effect of silver in chalcopyrite leaching may also be related to electrochemical modification whereby Ag_2S precipitates, which are transiently formed during leaching, establish cathodic areas which promote galvanic coupling. By comparison with elemental sulfur, silver sulfide granules have better conductivity and improved electron transport properties.

Wan *et al.*, (1985) demonstrated a positive catalytic effect of graphite on chalcopyrite leaching. These authors suggested that aggregates of $CuFeS_2$ and C (graphite) improved electron conductivity, thereby initially enhancing the solubilization of chalcopyrite. The rate eventually becomes limited owing to the formation of the semiconductive elemental sulfur layer. The catalyst effect varied depending on the conductivity, particle size, and the relative $CuFeS_2/C$ ratio of the pulp (Wan *et al.*, 1984, 1985).

In the present work, the catalytic effect of silver addition was evaluated. Graphite was also tested as a catalyst for the biological leaching of chalcopyrite. Evaluation in shake flasks leaching studies was undertaken with two fundamentally different kinds of chalcopyrite ore samples; one contained pyrite and the other contained pyrrhotite as the predominant iron sulfide.

MATERIALS AND METHODS

The ore samples originated from two different sulfide mineralizations. Sample A represented a chalcopyrite-pyrite ore material which also contained sphalerite. Chemical analysis yielded a partial elemental composition of 30.5% Fe, 2.6% Cu, 3.5% Zn, and 41.6% S. The sample was ground and sieved to a -200 to +325 mesh size fraction and used as a 5% suspension in shake flask experiments. The sample contained approximately 75% pyrite and 6% chalcopyrite. Mineralogical examination indicated the absence of pyrrhotite in this ore sample.

Sample B contained chalcopyrite-pyrrhotite as major sulfide minerals, with a minor amount of pyrite and sphalerite. Chemical analysis yielded the following partial composition: 15.8% Fe, 4.4% Cu, 0.05% Zn, and 11.2% S. The sample was ground and used as a -200 to +325 mesh sieve fraction at 5% pulp density. Pyrrhotite and pyrite accounted for approximately 23% and 1%, respectively, of the total mineral content. About 13% of the sample was chalcopyrite.

Shake flasks were inoculated with a mixed culture which was originally enriched for using a sulfide ore sample as the substrate. The medium contained 0.4 g per liter each of K_2HPO_4, $(NH_4)_2SO_4$, and $MgSO_4 \cdot 7H_2O$ at pH 2. Sulfuric acid was used for pH adjustment. For leaching experiments, the culture was first grown with a pyrite concentrate as the substrate. Cells was harvested by centrifugation and resuspended in the mineral salts solution before inoculation. The inoculum was standardized to $3.8 \cdot 10^9$ cells for each 250 ml culture. The cell suspension was standardized by microscopic counts. Silver sulfate was added to a final concentration of 9 and 30 ppm Ag^+ after 18 days (sample A) or 5 days (sample B) of incubation. Graphite (-50 mesh) was added at the beginning of the experiment at concentration of 0.3% and 0.5% (wt/vol). The flasks were incubated on a gyratory shaker (180 rev per min) at 28°C.

The pyritic samples received a second inoculum of $2.8 \cdot 10^9$ cells after eleven days of incubation. Additionally, the leach solutions of the pyritic ore samples were replaced with fresh mineral salts medium (pH 2) on day 40.

Metals in leach solution samples were analyzed by atomic absorption spectrometry. Redox potential measurements are given relative to calomel reference electrode. Evaporation was determined by weight loss and compensated for by adding sterile distilled water.

RESULTS AND DISCUSSION

The microbiological leaching of copper from the pyritic ore sample is shown in Figure 1. The yield of leaching was about 21% after 45 days. In contrast, only about 6% yield of copper leaching was achieved with the pyrrhotite-containing ore sample (Figure 2). This difference may be partially attributed to the pyrite effect which, due to galvanic coupling, enhances the oxidative dissolution of chalcopyrite. Because pyrrhotite has a much lower rest potential compared with pyrite, an enhancing effect of galvanic coupling is thermodynamically not feasible with pyrrhotite/chalcopyrite couple.

For the first two weeks, pH adjustments were made to neutralize acid demand associated with the pyritic sample. Subsequently, the leaching became a net acid producing reaction, necessitating periodic base addition to maintain the acidity values were in the range of pH 1.2-1.3. The pyrrhotite sample displayed acid demand during the first two weeks and subsequently the pH remained in the range 1.75-1.9. for the duration of the time course. These relative differences in the pH changes are in keeping with the recognition that pyrite oxidation is an acid-producing reaction and pyrrhotite oxidation is an acid-consuming reaction. These differences were somewhat masked by other concurrent reactions which either produced (*e.g.*, hydrolysis of Fe^{3+}) or consumed (*e.g.*, Fe^{2+} oxidation) H^+.

Silver was added at 9 and 30 ppm Ag^+ on day 18 to the pyritic ore sample (180 and 600 mg Ag^+ per kg of the ore sample, respectively). Following the addition of 9 ppm Ag, the leaching of copper continued without the characteristic decline that occurred in the reference culture without silver (Figure 1). With 30 ppm Ag, the rate of copper leaching transiently increased. In both instances, the silver addition was accompanied by a decrease of 100-150 mV in redox potential. As the redox potential values increased to the preceding levels, the accelerating effect of silver was typically exhausted.

The positive effect was more pronounced with the pyrrhotitic ore samples which received silver after 5 days of incubation. With 30 ppm Ag, the rate of chalcopyrite solubilization increased within two days and levelled off by day 9 (Figure 2), at which time the redox potential had increased from +400 mV to a steady value of +610 mV. The subsequent rate was similar to that determined in the reference culture (without silver). Compared with the pyritic sample, the proportional increase in the rate and yield of copper leaching was more pronounced with the pyrrhotite ore sample.

Figure 3 shows the effect of graphite on the leaching of copper from the pyritic ore sample. With 0.5% (wt/vol) graphite, copper leaching clearly increased over the reference level. With the pyrrhotite sample, the addition of graphite did not result in as much change as it did in the case of the pyritic sample (Figure 4). Graphite slightly decreased the rate at which the redox potential was increasing, but the end values were about 610 mV with both samples. Thus these experiments indicated that graphite had slight inhibitory effect on ferrous iron oxidation.

The redox potential is greatly determined by the degree of iron oxidation in leach solutions. Bacterially mediated ferrous iron oxidation typically increases the redox potential to over 600 mV range. Should iron remain in the ferrous state, the resulting redox potential has a low value of about 400-450 mV in these experiments. Thus, the decreases in redox potential caused by silver addition reflect inhibition of ferrous iron oxidation. Silver is generally recognized as an inhibitor of bacterial iron oxidation but its effective concentration is greatly influenced by the formation of soluble and insoluble complexes (Tuovinen *et al.*, 1985).

The apparent inhibition of iron oxidation was only transient in silver and graphite treated samples. The solubility of silver in leach solutions containing sulfide minerals is limited (<0.1 ppm) (Ahonen and Tuovinen, 1990b); similarly, only trace concentrations of Ag^+ were found in leach solution samples in the present work. Thus the ensuing high redox potential can be construed to represent the resumption of bacterial iron oxidation upon Ag precipitation. Information on the toxicity of graphite to acidophilic thiobacilli in leach systems is not available at the present time. In view of its known properties and role in acid leaching, it can be concluded that graphite participates by improving electron conductivity and by sorption of metals, but these effects on microorganisms are not well understood. In view of its known properties and role in acid leaching, it can be conductivity and by sorption of metals, but these effects on microorganisms are not well understood.

Catalytic effects of graphite and silver were negligible in the absence of bacteria.

Graphite effects hold some promise for research and delopment of mineral leaching processes especially with pyrite-dominated chalcopyrite ores. With the pyritic sample, the catalytic effect of graphite was of the same magnitude as that observed for silver but persisted for a longer period of time. While at the present time neither silver nor graphite-catalyzed processes are feasible for commercialization of the bacterial leaching of chalcopyrite containing ore materials or concentrates, the catalyst effects may provide a useful tool in future work in studying the electrochemistry of biological leaching processes.

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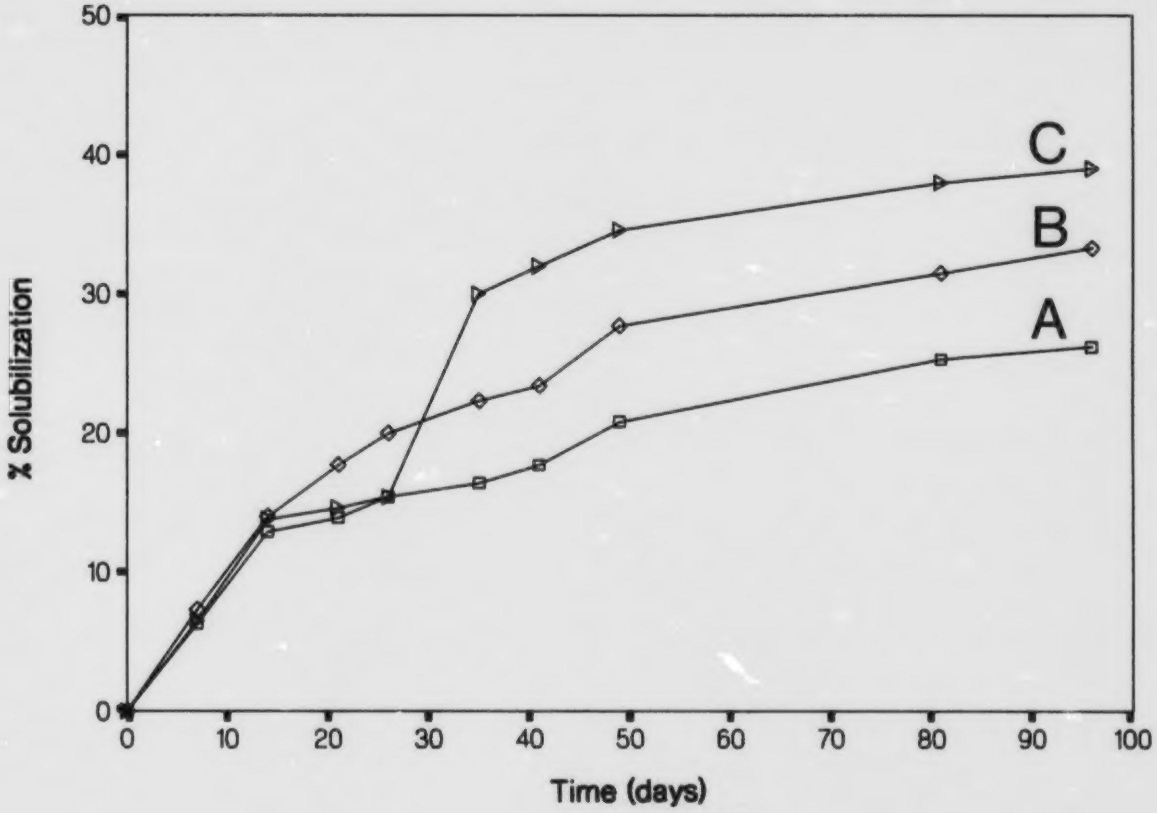

Fig. 1 Effect of silver on copper solubilization in the bacterial leaching of the pyrite chalcopyrite ore sample. The cultures received silver sulfate on day 18 as follows: A, no addition; B, 9 ppm Ag^+; C, 30 ppm Ag^+.

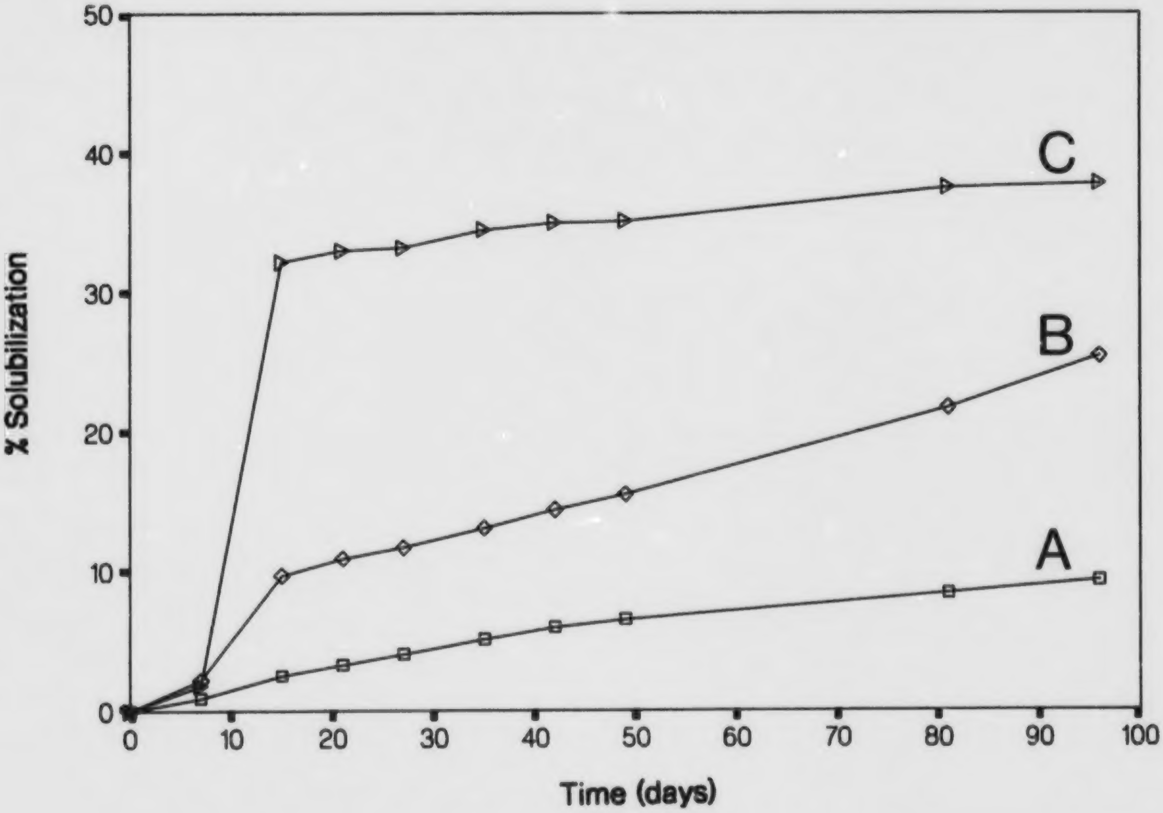

Fig. 2 Effect of silver on copper solubilization in the bacterial leaching of the pyrrhotitic chalcopyrite ore sample. the cultures received silver sulfate on day 5 as follows: A, no addition; B, 9 ppm Ag^+; C, 30 ppm Ag^+.

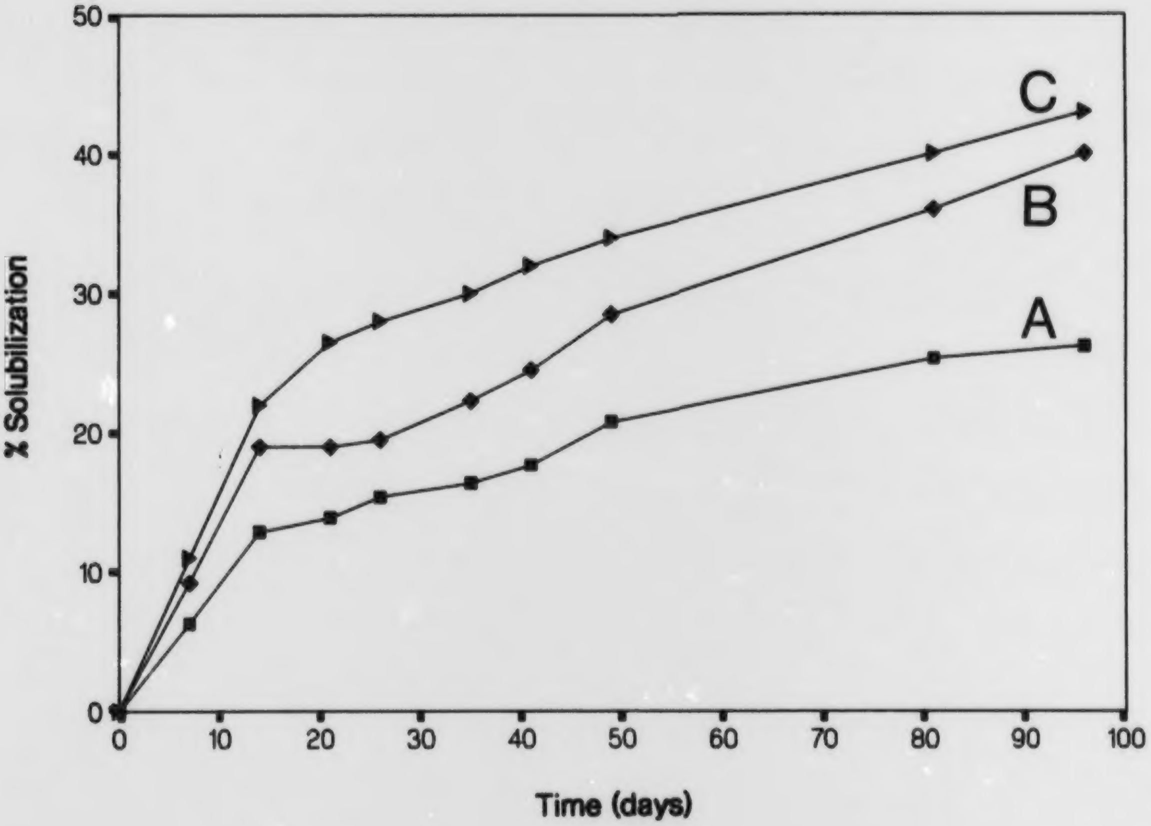

Fig. 3 Effect of graphite on copper solubilization in the bacterial leaching of the pyrite chalcopyrite ore sample. The cultures were initially amended with graphite as follows: A, no graphite; B, 0.35 (wt/vol) graphite; C, 0.5% (wt/vol) graphite.

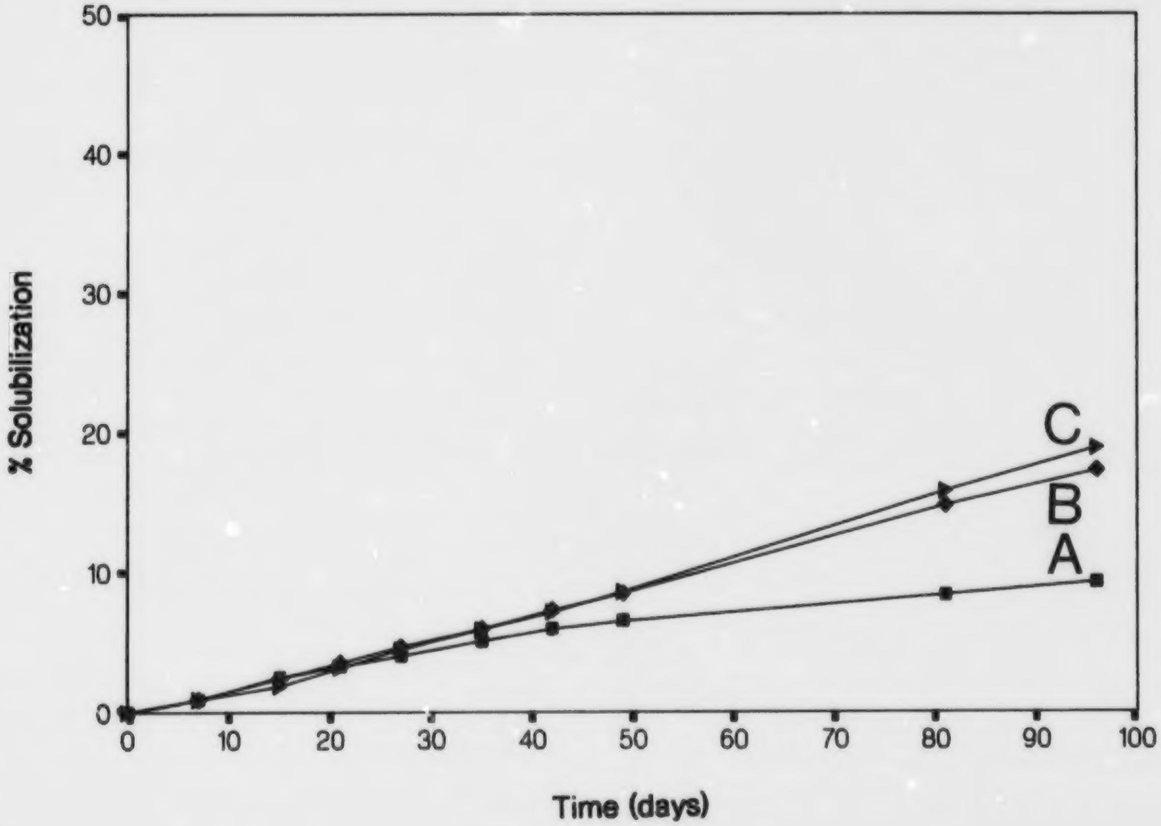

Fig. 4 Effect of graphite on copper solubilization in the bacterial leaching of the pyrrhotitic chalcopyrite ore sample. The cultures were initially amended with graphite as follows: A, no graphite; B, 0.3% (wt/vol) graphite; C, 0.5% (wt/vol) graphite.

ELECTROCHEMICAL ASPECTS OF PYRITE OXIDATION BY THIOBACILLUS FERROOXIDANS DURING LEACHING OF A CANADIAN URANIUM ORE

by

LUIS M. CHIA[1], *WAN K. CHOI*[1], *ROGER GUAY*[2]
AND ARPAD E. TORMA[1]

ABSTRACT

Using a cyclic voltammetric technique it was demonstrated that uranium leaching from a low-grade ore containing pyrite is a complex phenomenon. Many intermediate electrochemical reactions are involved in the oxidation of solid semiconductor pyrite, which is the source of oxidant (Fe^{3+}) for uranium. The oxidation of pyrite was found to be controlled by solid-state diffusion, as suggested by chronoamperometric and chronopotentiometric measurements. Using the Sand's method, the activation energy of solid-state diffusion was calculated to be $\Delta Ea = 22.4$ kJ. From the effect of pulp density of uranium ore, the maximum rate of uranium extraction by *Thiobacillus ferrooxidans* was calculated from a modified Monod equation to be $V_m = 1.37 \times 10^{-6}$ M s^{-1}. A generalized model for the mechanism of pyrite oxidation is presented.

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[1] Department of Materials and Metallurgical Engineering, New Mexico Institute of Mining and Technology, Socorro, NM 87801.

[2] On sabbatical leave from Laval University, Quebec, Que., Canada G1K 7P4.

ASPECTS ÉLECTROCHIMIQUES DE L'OXYDATION DE LA PYRITE PAR *THIOBACILLUS FERROOXIDANS* PENDANT LA LIXIVIATION D'UN MINERAI D'URANIUM CANADIEN

L. M. Chia[1], W. K. Choi[1], R. Guay[2] et A. E. Torma[1]

RÉSUMÉ

L'utilisation d'une technique voltamétrique cyclique a démontré que la lixiviation d'Uranium à faible teneur contenant de la pyrite est un phénomène complexe. De nombreuses réactions électrochimiques intermédiaires interviennent dans l'oxydation de la pyrite, semi-conducteur solide comme source d'oxydant (Fe^{3+}) pour l'uranium. On a déterminé que l'oxydation de la pyrite était fonction d'une diffusion à l'état solide, comme le suggèrent les mesures chrono-ampérométriques et chrono-potentiométriques. Selon la méthode Sand, l'énergie nécessaire pour permettre la diffusion à l'état solide a été calculée comme étant E_a = 22,4 kJ. Compte tenu de la masse volumique du minerai d'uranium, la valeur maximale de la vitesse d'extraction de l'uranium par *Thiobacillus ferrooxidans* a été calculée à l'aide d'une équation modifiée de Monod comme étant $V_m = 1.37 \times 10^{-6}$ M s^{-1}. Un modèle généralisé du mode d'oxydation de la pyrite est présenté.

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[1] Department of Material and Metallurgical Engineering New Mexico Institute of Mining and Technology Socorro, NM E.-U.

[2] En congé sabbatique de l'Université Laval Québec (Québec) Canada

INTRODUCTION

Bacterial extraction of uranium from diverse ores has been studied by many investigators in the last 35 years (Fisher, 1966; Duncan and Bruynesteyn, 1971; Guay *et al.*, 1977; Tomizuka and Yagisawa, 1978; Agate, 1983; Torma, 1985; Livesey-Goldblatt, 1986; McCready, 1988). In this process, the insoluble tetravalent uranium is oxidized by ferric ion to a soluble hexavalent uranium species. Ferric iron (oxidant) is obtained by bacterial oxidation of pyrite which is always associated in varying amounts with the uranium ore. The overall reaction of pyrite oxidation is given by:

$$2FeS_2 + 7.5\ O_2 + H_2O \xrightarrow{\text{bacteria}} Fe_2(SO_4)_3 + H_2SO_4 \qquad (1)$$

Reaction 1 is well documented (Lowson, 1982 and Guay *et al.*, 1989). It is the sum of anodic oxidation of pyrite and cathodic reduction of elemental oxygen which is dissolved from air during aeration of the leach solution. The success of uranium leaching is dependent to a large extent on the efficiency of microorganisms in oxidizing pyrite. However, the metabolic energy (in form of electrons) from pyrite oxidation is influenced by the inhomogeneity of the mineral surface, dissolved oxygen concentration, the iron redox cycle and varying concentrations of ferrous, ferric, heavy metal (impurities) and uranyl ions, which are released during the leaching process. The metabolic activity is further complicated by the fact that pyrite is a semiconductor, showing a lack of stoichiometry between its iron and sulfur contents. Pyrite may occur naturally in n- and p-types. The n-type pyrite has excess of free electrons and the p-type excess of positive charge (or holes). Their resistivity to conduct electricity is 1×10^{-3} and 3×10^{-2} ohm.m, respectively (Crundwell, 1988). Pyrite has a cubic crystallographic structure similar to NaCl and its ionic model can be written as $Fe^{2+}(S_2)^{2-}$ (Prewitt and Rajamani, 1974; Vaughan and Craig, 1978). As shown in Fig. 1, the lower part of the conduction band in pyrite is derived from the anti-bonding e_g* doublet of 3d and upper part of 4s orbitals of iron, while the valence band is derived from the t_{2g} triplet of the 3d orbital of iron and 2pπmolecular orbital of the $(S_2)^{-2}$ group (Vaughan and Craig, 1978). The σmolecular orbital can be formed by overlaps of s-p and p-p atomic orbitals, and the πmolecular orbital by d-p and d-d atomic orbitals. The valence band in pyrite is mainly due to 3p and 3s orbitals of sulfur while the conduction band is mainly due to 4p and 4s orbitals of iron. Therefore, the conduction in pyrite takes place when electrons are exited into the e_g* orbital. The sp^3 represents the hybrid molecular orbital of sulfur and the 1 eV energy between the non-bonding t_{2g} and antibonding e_g* orbitals (Ennaou *et al.*, 1986). Pure pyrite is known to resist chemical dissolution because its valence band is of a non-bonding character, and the holes do not contribute to bond breaking (Crundwell, 1988).

The present study investigated the intermediate reactions involved in the bacterial leaching of a pyritic uranium ore using adapted culture of *Thiobacillus ferrooxidans* and electrochemical techniques, such as cyclic voltammetry, chronoamperometry and chronopotentiometry.

MATERIALS AND METHODS

Bacteria

A culture of *T. ferrooxidans* was routinely maintained in our laboratories on a modified nutrient medium, in which the energy source, ferrous sulfate, was replaced by a pyritic uranium ore (Chia, 1983). When bacterial growth reached the late logarithmic phase, a portion of the suspension was transferred into a fresh medium to maintain the stock culture to be or used as an experimental inoculum.

Uranium Ore

The ore used in this investigation was obtained from Rio Algom Company, Ltd., Ontario, Canada. It was ground to particle size less than -38 μm and contained 5.02% pyrite and 1,123 ppm UO_2 in addition to 83.6% SiO_2, 6.7% Al_2O_3 and other minor constituents.

Bioleaching Experiments

This series of experiments was carried out in 250 cm^3 Erlenmeyer flasks pulp density, containing varying amounts of ore to yield 5 to 30%, PD suspensions, 70 cm^3 iron free nutrient medium (Chia, 1983) and 5 cm^3 inoculum of *T. ferrooxidans*. In the sterile controls the inoculum was replaced by an equal volume of a 2% thymol (in alcohol) solution. The flasks were incubated at 30°C and 250 rpm on a New Brunswick gyratory incubator shaker, model G26. Samples were periodically removed from the leach solutions and analyzed on a Princeton Gamma-Tech chemical analyzer, model 100.

Electrochemical Measurements

All these experiments (cyclic voltammetry, chronoamperometry and chronopotentiometry) were done in 200 cm^3 electrochemical cells each equipped with a FeS_2-graphite paste (working) electrode, a platinum (counter) electrode and a saturated calomel (reference) electrode for Eh and pH measurements, temperature control, a potentiostat (Pine Instrument Co., Model RDE4), and an X-Y recorder (EG&G Princeton Applied Research, Model RE 0092). The cells were charged with 150 cm^3 leach suspensions (inoculated or sterile) at the start of each experiment. The pyrite-graphite paste electrode consisted of a layer of graphite paste (which is composed 5 g graphite and 2 cm^3 paraffin oil) at the bottom of the electrode, upon which a homogenized layer of FeS_2-graphite paste was added. The composition of this latter paste was 1 g FeS_2, 4 g graphite powder and 2 cm^3 paraffin oil. A platinum wire was inserted into the pastes. A diagram of the experimental setup and that of the carbon paste electrode is shown elsewhere (Choi and Torma, 1989).

RESULTS AND DISCUSSION

Typical leach curves of uranium extraction for 16% pulp density suspensions in the presence and absence of bacteria are shown in Fig. 2. Bacterial efficiency is demonstrated by the difference between the inoculated and sterile control experiments. The kinetic values [V(mg dm^{-3} h^{-1}) and Y(%)] were calculated as shown in Fig. 3. Uranium extraction can be expressed by:

$$UO_2 + Fe_2(SO_4)^3 + 2H_2SO_4 \rightarrow H_4[UO_2(SO_4)_3] + 2FeSO_4 \qquad (2)$$

Ferric sulfate is obtained from bacterial oxidation of pyrite as indicated in equation 1. Ferrous sulfate set free in reaction 2 is oxidized to ferric sulfate by the bacteria:

$$2FeSO_4 + H_2SO_4 + 0.5\ O_2 \xrightarrow{\text{bacteria}} Fe_2(SO_4)_3 + H_2O \qquad (3)$$

Uranium is dissolved in sulfato-complex form and after solid-liquid separation, uranium can be extracted from the filtrate by solid ion exchange resins. The maximum theoretical rate, V_m, and the K-value were determined from the data derived in a series of pulp density, (PD), experiments (PD was varied from 2 to 32%) using a linearized form of Monod equation (Guay *et al.*, 1989):

$$V = V_m - K\frac{V}{PD} \qquad (4)$$

Figure 3 is a plot of V vs V/PD. The slope of the straight line corresponds to -K, the intercept with the V-axis to V_m, and the intercept with the V/PD-axis to V_m/K. As a consequence, the rate of uranium extraction (Ms^{-1}) can be expressed as function of pulp density by:

$$V = 1.37 \times 10^{-6}\frac{PD}{7.45 + PD} \qquad (5)$$

The highest experimental rate value of uranium extraction was 1.11 Ms^{-1}, obtained with a 32% pulp density suspension, while the highest yield, Y, of extraction was about 87%, achieved with leach suspensions of 28 and 32% PD.

The difference in the cyclic voltammograms of inoculated and sterile experiments is shown in Fig. 4. The curves represent the initial experimental conditions. The relative increase in the reaction currents in the inoculated experiment is due to the presence of small amounts of iron transferred with the inoculum into the experimental solution. If during the oxidation of pyrite all the electron transfer processes ($Fe^{2+} \rightarrow Fe^{3+} + e$; and $S^{2-} \rightarrow S^{6+} + 8e$) would take place in one single reaction then only one oxidation peak should have been realized during the positive sweep and one reduction peak during the reversal of the potential (negative sweep). Since Fig. 4 shows a number of oxidation (B, C, D and E) and reduction (A, F, G, H and I) peaks, it is concluded that intermediate reactions occured during pyrite electrochemical treatments. It is important to note that while the cyclic voltammetry detects the existence of these reactions as indicated by the peaks, the peak reactions remain to be specified by the investigators by using thermodynamic data available from the literature. The half-peak potential ($E_{p/2}$) must be estimated from the experimental curves and then converted to potentials measured against a standard hydrogen electrode (SHE). These latter potentials correspond to the normal decomposition potential (E°) that can be calculated for the proposed peak reaction.

$(E_{p/2})$ vs SCE$)_{expr}$ conversion $(E_{p/2}$ vs SHE$)_{expr} \simeq E^{\circ}$ calculated (6)

The following electrochemical reactions can be proposed for the peaks in Fig. 4:

Peak A (reduction of pyrite):

$FeS_2 + 2e \rightarrow FeS + S^{2-}$; $E^{\circ} = -0.75$ V (7)
$E_{p/2} = -0.70$ V vs SCE = -0.46 V vs SHE

Peak B (oxidation of H_{2s}):

$H_2S \rightarrow S^{\circ} + 2H^+ + 2e$; $E^{\circ} = 0.14$ V (8)
$E_{p/2} = 0.00$ V vs SCE = 0.24 V vs SHE

Peak C (oxidation of FeS_2):

$FeS_2 + 8H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 16H^+ + 14e$; $E^{\circ} = 0.36$ V (9)
$E_{p/2} = 0.14$ V vs SCE = 0.38 V vs SHE

or

$FeS_2 \rightarrow Fe^{2+} + 2S^{\circ} + 2e$; $E^{\circ} = 0.34$ V (10)
$E_{p/2} = 0.14$ V vs SCE = 0.38 V vs SHE

Peak D (possible oxidation reactions):

a) $FeS_{(2-x)} \rightarrow Fe^{2+} + (2-x)S^{\circ} + (2-x)e$ (11)
$E_{p/2} = 0.20$ V vs SCE = 0.42 V vs SHE

b) $S^{\circ} + 4H_O \rightarrow SO_4^{2-} + 8H^+ + 6e$; $E^{\circ} = 0.36$ V (12)
$E_{p/2} = 0.20$ V vs SCE = 0.42 V vs SHE

c) $S^{2-} + 4H_2O \rightarrow SO_4^{2-} + 8H^+ + 8e$; $E^{\circ} = -0.15$ V (13)
$E_{p/2} = 0.20$ V vs SCE = 0.42 V vs SHE

d) $Fe^{2+} \rightarrow Fe^{3+} + e$; $E^{\circ} = 0.77$ V (14)
$E_{p/2} = 0.20$ V vs SCE = 0.42 V vs SHE

Peak E (ferrous ion oxidation):

$Fe^{2+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^+ + e$; $E^{\circ} = 1.05$ V (15)
$E_{p/2} = 0.76$ V vs SCE = 1.00 V vs SHE

Peak F (reduction of ferric hydroxide):

$Fe(OH)_3 + 3H^+ + e \rightarrow Fe^{2+} + 3H_2O \quad ; \quad E^\circ = 1.05\ V$ (16)
$E_{p/2} = 0.75$ V vs SCE = 0.99 V vs SHE

Peak G (possible reduction reactions):

a) $Fe^{2+} + (2\text{-}x)S^\circ + (2\text{-}x)e \rightarrow FeS_{(2\text{-}x)}$ (17)
$E_{p/2} = 0.25$ V vs SCE = 0.49 V vs SHE

b) $Fe^{2+} + (2\text{-}x)\ SO_4^{2-} + 8(2\text{-}x)H^+ + (14\text{-}6x)e \rightarrow FeS_{(2\text{-}x)} + 4(2\text{-}x)H_2O$ (18)
$E_{p/2} = 0.25$ V vs SCE = 0.49 V vs SHE

c) $SO_4^{2-} = 8H^+ + 6e \rightarrow S^\circ + 4H_2O \quad ; \quad E^\circ = 0.36\ V$ (19)
$E_{p/2} = 0.25$ V vs SCE = 0.49 V vs SHE

d) $SO_4^{2-} + 8H^+ + 8e \rightarrow S^{2-} + 4H_2O \quad ; \quad E^\circ = -0.15V$ (20)
$E_{p/2} = 0.25$ V vs SCE = 0.49 V vs SHE

Peak H (formation of pyrite):

$Fe^{2+} + 2SO_4^{2-} + 16\ H^+ + 14e \rightarrow FeS_2 + 8H_2O \quad ; \quad E^\circ = 0.36\ V$ (21)
$E_{p/2} = 0.07$ V vs SCE = 0.31 V vs SHE

Peak I (reduction of elemental sulfur):

$S^\circ + 2H^+ + 2e \rightarrow H_2S \quad ; \quad E^\circ = 0.14\ V$ (22)
$E_{p/2} = 0.10$ V vs SCE = 0.34 V vs SHE

When the cyclic voltammograms were repeated several times during the first 20 minutes, the peak currents were decreased as shown for the inoculated experiments in Fig. 5. The decrease in the peak current is the consequence of disappearance of active sites from the surface of FeS_2-carbon paste electrode.

In the potential range of 0.0 to 0.2 volt, the reversible peaks B, C, F and G correspond to the electroadsorption and desorption of protons onto the surface layers of pyrite, which is in good agreement with the findings of other investigators (Mishra and Osseo-Assare, 1988a). Pyrite oxidation at higher potentials occurs by oxidation of hydroxyl ion by Fe 3d states followed by transfer of OH- to $(S^2)^{2-}$ state, as suggested by the peaks D and E. The presence of thiosulfate was reported to occur during these transformations (Mishra and Osseo-Assare, 1988b) in acidic media. During pyrite oxidation, non-stoichiometric sulfide layers may be formed as shown for peaks D and G. Similar findings were reported for the oxidation of pyrite (Buckley *et al.*, 1988). However, it is expected that significant differences will be observed in the oxidation behaviour of pyrite from different sources.

During the leaching, considerable amounts of ferrous, ferric and uranyl ions are dissolved from the ore. Their effects on the initial peaks on cyclic voltammogram were studied with individual leach suspensions containing varying amounts of Fe^{2+}, Fe^{3+} and UO_2^{2+} ions, as shown in Fig. 6.

The nature of pyrite dissolution (chemically or diffusion controlled) was studied by chronopotentiometric and chronoamperometric techniques in presence and absence of microorganisms. The chronopotentiometric experiments were carried out in the range of constant currents of 0.025 to 0.115 $mA{\cdot}cm^{-2}$. A typical chronopotentiometric curve is shown in Fig. 7A, indicating the determination of transition time τ(sec). Figure 7B summarizes the chronopotentiometric data in terms of plots of $i\tau^{1/2}$ versus constant currents i. In this figure, it can be seen that constant current densities at higher than 0.07 $mA{\cdot}cm^{-2}$, the $i\tau^{1/2}$ term becomes independent of a further increase in i, indicating that pyrite dissolution is controlled by solid-state diffusion phenomena (Bard and Faulkner, 1980; Price, 1981). This finding has been verified by chronoamperometric measurements which were carried out at constant applied cell potentials at 500, 650 and 800 mV by observing the change in the reaction currents, i, as a function of time (t). A typical example of chronoamperometric curves is shown in Fig. 8. At predetermined times the reaction current is read from the i-axis, then $it^{1/2}$ calculated and plotted against t, as shown in picture B. The curves shown in Fig. 8B are independent of time after 10 sec. This behaviour is typical for reactions controlled by solid-state diffusion (Bard and Faulkner, 1980; Price, 1981). If the chronopotentiometric measurements are carried out at different temperatures, the activation energy (ΔE_a) of solid-state diffusion can be calculated using Sand's technique (Price, 1981). Figure 9A is a plot of $i\tau^{1/2}$ vs i at temperature varying from 21 to 73°C. The $i\tau^{1/2}$-values are constant at i-values higher than 0.12 $mA \cdot cm^{-2}$, which can be used to calculate ΔE_a.

$$i\tau^{1/2} = \tfrac{1}{2}\pi^{1/2} nFD^{1/2}C \qquad (23)$$

where n is the number of electrons transferred in the electrochemical reaction, F is the Faraday's constant, C is the concentration of pyrite and D is the solid-state diffusion coefficient, which is defined as:

$$D = D_o \exp(-\Delta E_a/RT) \qquad (24)$$

where R is the gas constant and T the absolute temperature. Combination of equations 23 and 24 results in the following expression:

$$(i\tau^{1/2} = \tfrac{1}{2}\pi^{1/2} nFC[D_o \exp(-\Delta E_a/RT)]^{1/2})^2 \qquad (25)$$

By taking the natural logarithm of equation 25, we have

$$\ln(i^2\tau) = \ln(\pi n^2F^2D_oC^2/4) - \Delta E_a/RT \qquad (26)$$

A plot of ln $(i\tau^{1/2})$ vs 1/T gave a straight line (as shown in Fig. 9B) with slope equal to $\Delta E_a/R$ from which the apparent activation energy was calculated to be $\Delta E_a = 22.4$ kJ. This relatively low value of ΔE_a suggest that pyrite oxidation is controlled by solid-state diffusion, which is in excellent agreement with the interpretation of data in Fig. 7 and 8.

MECHANISM OF URANIUM EXTRACTION

The overall uranium extraction is specified in equation 2. Accordingly, the presence of ferric ion is required for the oxidation of UO_2 to UO_2^{2+}. To shed more light on the production of ferric ion, the pyrite oxidation mechanism must be explained. Figure 10 illustrates the schematic energy density of state (a) and band diagrams (b) of n-type pyrite in contact with nutrient solution. In the interface between the solid semiconductor pyrite and the 9K acidic nutrient solution, there are many ionic species which have different energy levels according to their work function (Bockris and Khan, 1985). If the free energy of electrons in the redox system, E_f(redox), of 9K nutrient solution is equivalent with the Fermi energy level (E_f) in solid FeS_2 semiconductor, then pyrite may be decomposed. In the n-type pyrite, the Fermi level is generally located close to the energy level of the conduction zone, where the electrons are mobile and easily removable. The relative position of E_f of a redox leach system to the decomposition energy level E_d of pyrite provides information about the chemical stability of pyrite. Therefore, n-type pyrite are easily oxidizable but microorganisms while the p-type pyrites are not. Note that in the p-type pyrite the E_f is located close to the energy level of the valence zone. Therefore the p-type is very stable and difficult to oxidized. As shown in Fig. 10, the lower energy level (valence) band is completely filled with electrons, while the high energy level (conduction) band is incompletely filled. Between these two zones is an energy gap which is also called the forbidden energy zone. When ferrous ion leaves the solid pyrite crystal an excess of negative ions, S^{2-}, is left behind. This will establish a potential difference between the solid pyrite and the nutrient solution that will tend to prevent the movement of Fe^{2+} ions into the bulk of the solution. These are not free immediately to move out of the crystal since their energy level is in the filled valency band. However, the movement of electrons may be assisted by holes (h^+), which were injected into t_{2g} a filled σvalence band by $^{\bullet}OH$ radicals. For example, by the following reaction:

$$H_2O + h^+ \rightarrow H^+ + {}^{\bullet}OH \qquad (27)$$

The ˙OH radical will react with the S^{2-} in the valency band by forming S° or SO_4^{2-} species. As a result, the movement of Fe^{2+} ions into the bulk of the nutrient leach solution will be facilitated. In the case of p-type pyrite, the excess charge on S^{2-} could be neutralized by transport of holes through the valence band, so that the movement of Fe^{2+} ions will be uninhibited. However, there is non-bonding t_{2g} in the valence band of FeS_2, and thus, the movement of Fe^{2+} into the solution remains restricted.

The mechanism of pyrite oxidation can be schematically presented as shown in Fig. 11. In the absence of bacteria the final product of pyrite oxidation is ferrous ion and elemental sulfur and in the presence of bacteria ferric and sulfate ions. For the purpose of simplicity, the intermediate products shown in the electrochemical studies are not indicated.

CONCLUSION

The applicability of electrochemical techniques in the elucidation of reactions involved in the bacterial leaching of a low-grade uranium ore containing pyrite has been demonstrated. The effect of the semiconductor character of pyrite on the leachability of uranium was discussed. It was found that pyrite oxidation is controlled by solid-state diffusion phenomena.

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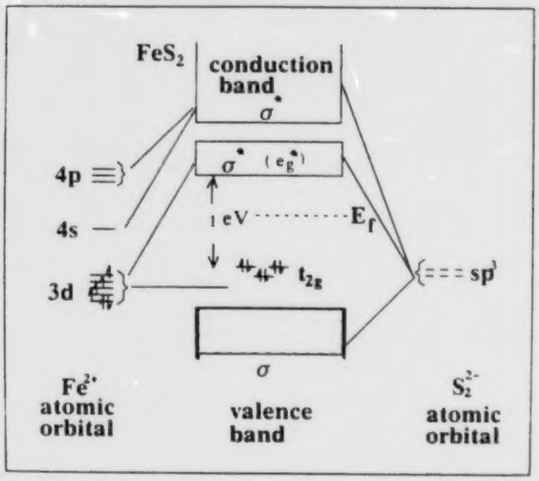
FeS₂
conduction band
σ*
4p
4s
3d
σ* (eg*)
1 eV
Ef
t2g
sp³
σ
Fe²⁺ atomic orbital
valence band
S₂²⁻ atomic orbital

Fig. 1 Schematic presentation of molecular orbital and band diagram for pyrite.

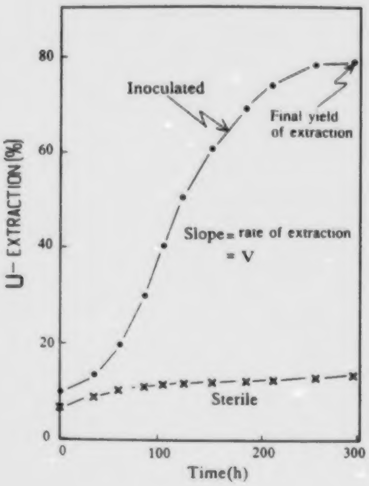

Fig. 2 Uranium extraction from a 16% pulp density suspension at 35°C and 250 rpm.

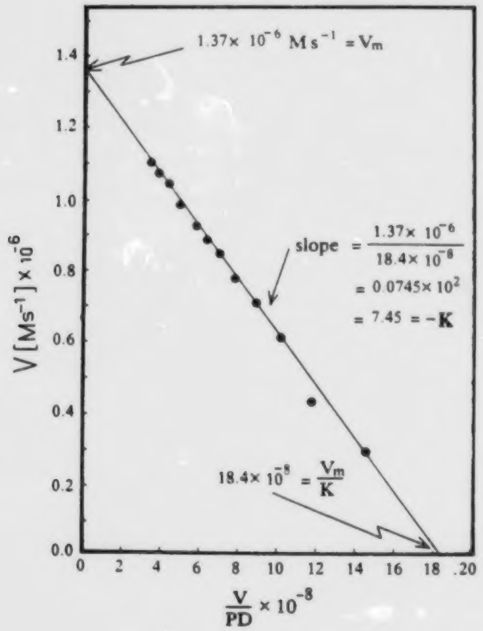

Fig. 3 Linearized Monod plot for the effect of pulp density.

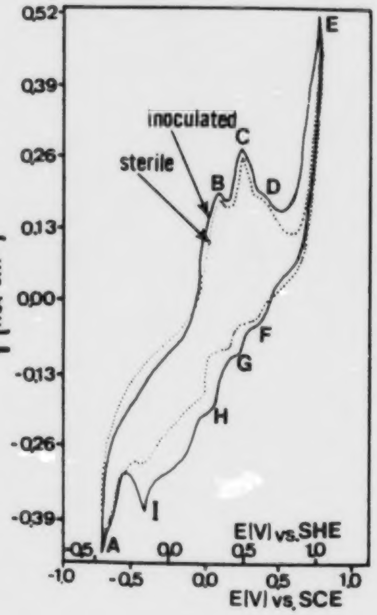

Fig. 4 Cyclic voltammograms with a 20% FeS_2-carbon paste electrode at 25°C and sweep rate of 100 mV s^{-1}.

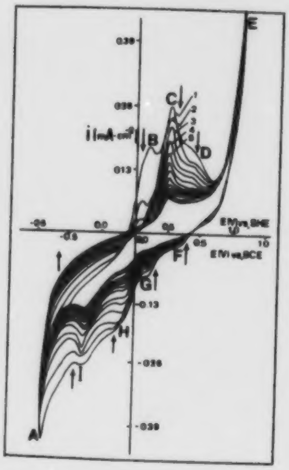

Fig. 5 Influence of time on the cyclic voltammogram of a 20% FeS2-carbon paste electrode at 25°C and sweep rate of 100 mV s^{-1}.

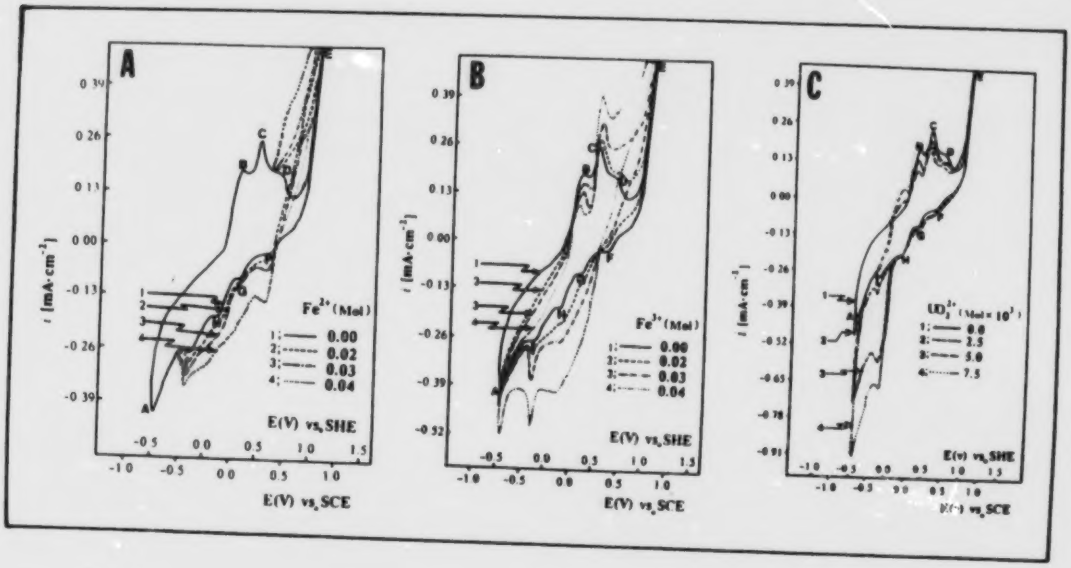

Fig. 6 Effects of ferrous (picture A), ferric (picture B) and uranyl (picture C) ion concentrations on the curves of cyclic voltammogram of a 20% FeS$_2$-carbon paste electrode at 25°C and sweep rate of 100 mV s^{-1}.

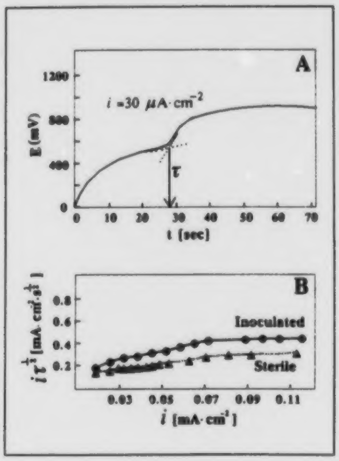

Fig. 7 A typical chronopotentiometric curve (Fig. 7A) of 20% FeS_2-carbon paste electrode in 9K nutrient medium in presence of *Thiobacillus ferrooxidans* at 35°C. Figure 7B represents the summary of chronopotentiometric data in terms of plots $i\tau^{1/2}$ versus i for the inoculated and sterile runs.

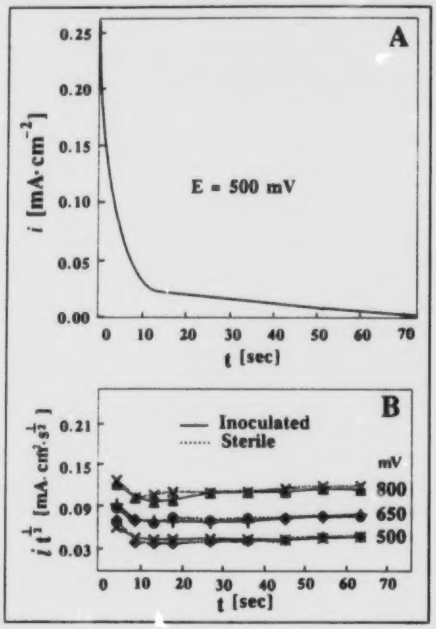

Fig. 8 A typical chronoamperometric curve is shown in picture A using 20% FeS_2-carbon paste electrode in 9K nutrient medium in presence of *Thiobacillus ferrooxidans* at 35°C. Picture B represents the summary of composite data in terms of $i\tau^{1/2}$ vs t.

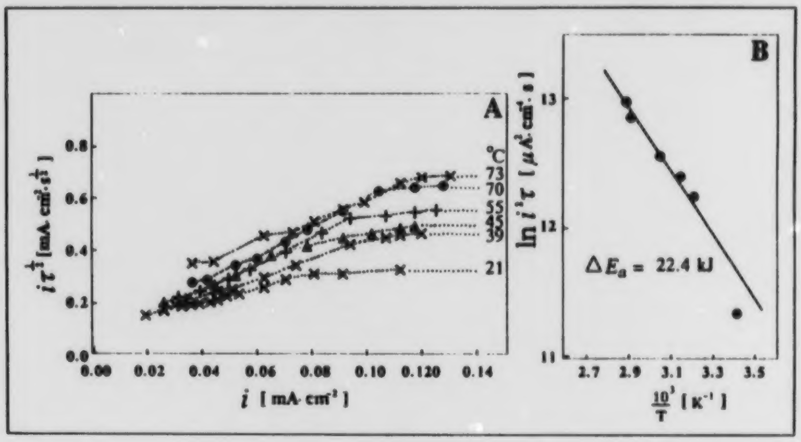

Fig. 9 Plots of $i\tau^{1/2}$ as a function of i (Fig. 7A) and Arrhenius type plot of $\ln(i\tau^{1/2})$ vs 1/T. Data are from chronopotentiometric measurements using 20% FeS_2-carbon paste electrode in 9K sterile medium at different temperatures.

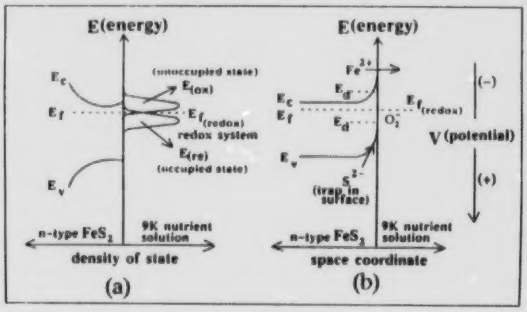
E(energy)
E_c
E_f
E_v
(unoccupied state)
$E_{(ox)}$
$E_{f(redox)}$
redox system
$E_{(re)}$
(occupied state)
n-type FeS_2
9K nutrient solution
density of state
(a)
E(energy)
Fe^{2+}
E_d
E_c
E_f
$E_{f(redox)}$
O_2^-
E_v
S^{2-}
(trap in surface)
(–)
V (potential)
(+)
n-type FeS_2
9K nutrient solution
space coordinate
(b)

Fig. 10 Schematic presentation of energy of density states (a) and band diagram (b) of n-type pyrite and acidic nutrient leach solution.

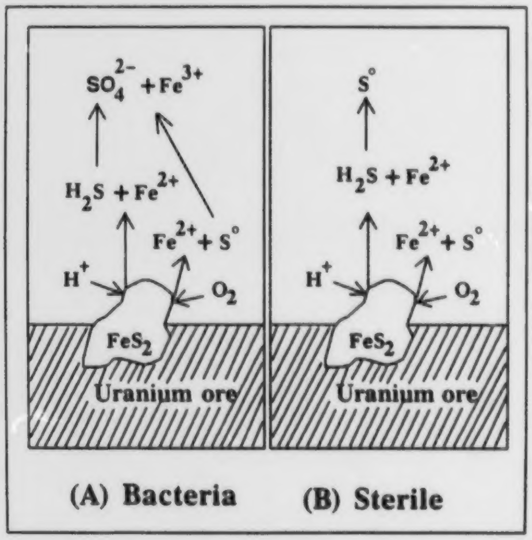
$SO_4^{2-} + Fe^{3+}$
$H_2S + Fe^{2+}$
$Fe^{2+} + S^\circ$
H^+
O_2
FeS_2
Uranium ore
(A) Bacteria
S°
$H_2S + Fe^{2+}$
$Fe^{2+} + S^\circ$
H^+
O_2
FeS_2
Uranium ore
(B) Sterile

Fig. 11 Oxidation of pyrite in the presence and absence of bacteria.

THE OXIDATION OF ARSENIC IN ARSENOPYRITE : THE TOXICITY OF As(III) TO A MODERATELY THERMOPHILIC MIXED CULTURE

by

J. BARRETT[1], *D.K. EWART*[2], *M.N. HUGHES*[1], *A.M. NOBAR*[1] *AND R.K. POOLE*[2]

ABSTRACT

Substantial concentrations of arsenic(III) have been detected during the bio-oxidation of a gold-bearing arsenopyrite concentration by a moderately thermophilic mixed culture. Concentrations of As(III) in the range 10-20 mM (0.75-1.5 g L^{-1} As) have been found routinely, while concentrations as high as 70-90 mM As(III) (5.25- 6.75 g L-1 As) have been present in the supernatant solutions from dead cultures. Oxidation of As(III) to As(V) could not be effected by Fe(III) alone but did occur in the presence of Fe(III) and a culture growing on pyrite. Cells which had been thoroughly washed to remove Fe(III) could not oxidize As(III).

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[1] Department of Chemistry.

[2] Division of Biosphere Sciences, King's College London, Campden Hill Road, London W8 7AH, U.K.

L'OXYDATION DE L'ARSENIC DANS L'ARSÉNOPYRITE : LA TOXICITÉ DE As(III) SUR UNE CULTURE MIXTE MODÉRÉMENT THERMOPHILE.

J. Barrett[1], D.K. Ewart[2], M.N. Hughes[1], A.M. Nobar[1] et R.K. Poole[2]

RÉSUMÉ

Des concentrations substantielles d'arsenic(III) ont été décelées pendant la bio-oxydation d'un concentré d'arsénopyrite aurifère par une culture mixte modérément thermophile. Des concentrations de As(III) de l'ordre de 10 à 20 μM (0,75 - 1,5 g L^{-1} AS) ont été décelées systématiquement, alors que des concentrations pouvant atteindre de 70 à 90 μM AS(III) (5,25 - 6,75 g L-1 As) étaient présentes dans le surnageant de cultures mortes. La transformation de As(III) en As(V) sous l'effet de l'oxydation ne pouvait être réalisée uniquement en présence de Fe(III), mais elle s'est produite en présence de Fe(III) et d'une culture se développant sur la pyrite. Les cellules à partir desquelles Fe(III) a été entièrement éliminé par lavage, ne pouvaient pas oxyder As(III).

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[1] Département de Chimie

[2] Division des Sciences de la Biosphère, King's College London, London, U.K.

INTRODUCTION

There is much current interest in the bio-oxidation of gold-bearing arsenopyrite concentrates to allow the enhancement of gold recovery by cyanidation. This process offers a viable alternative to roasting or pressure oxidation on economic considerations, while roasting has severe environmental disadvantages. We have successfully used a moderately thermophilic mixed culture to catalyzes the oxidation of a gold-bearing arsenopyrite concentrate from Western Australia, which contains about 14% arsenic by weight. Treatment of this material results in the solubilization of arsenic compounds. Using an arsenic-conditioned culture, we have successfully worked at pulp densities which give about 190 mM (15 g L^{-1}) of arsenic in the medium. Under these conditions, the culture grows vigorously (Barrett *et al.*, 1988a). At higher pulp densities a deterioration in growth occurs, which may be attributed to arsenic toxicity. However, in experiments in stirred batch and continuous reactors, the onset of toxicity was sometimes sudden and was accompanied by an increase in pH of the medium. This toxicity was associated with the build up of high levels of arsenic (III) species in the medium. The presence of As(III) amongst the products of bio-oxidation of arsenopyrite is known, although no quantitative data are available. Shrestha (1988) has claimed that the arsenic in arsenopyrite is oxidized biologically to As(III), and that the As(III) is then chemically oxidized to As(V) by Fe(III). However, attention has not been drawn previously to the occurrence of such high levels of As(III) in reactors and the implications in terms of toxicity to the culture. As(III) is more toxic than As(V) to both Gram-positive and Gram-negative bacteria (Silver *et al.*, 1981). Bacterial resistance to As(III) and to As(V) is governed by separate genes, although in both cases the resistance mechanism involves ATP-driven efflux of the anion (Silver *et al.*, 1989).

We now report studies on the extent of formation of As(III) and on the pathways for the oxidation of arsenic (III) during the bio-oxidation of arsenopyrite, and also on the relative toxicities of As(III) and As(V) to the moderately thermophilic mixed culture. We have also investigated whether the oxidation of As(III) can be used as a source of energy by the culture, although earlier reports suggest that this is not the case for *Thiobacillus ferrooxidans* (Braddock *et al.*, 1984) and for an As(III)-oxidizing, acidiphilic bacterium (Wakao *et al.*, 1988).

Under the acidity conditions employed in the present work, As(III) is present as arsenious acid ($HAsO_2$, $pK_1 = 8.8$) and As(V) as arsenic acid (H_3AsO_4, $pK_1 = 2.4$).

EXPERIMENTAL

Culture and Bio-Oxidation Experiments

The growth characteristics of the culture have already been described (Barrett *et al.*, 1988b). All experiments were carried out in the weak medium of Norris (pers. commun., 1984). A mixture of air and CO_2 (1.0%) saturated with water was passed through each culture for the duration of the experiment. Experiments were carried out in shaken flasks or stirred 1-L reactors (described by Barrett *et al.*, 1988a) as noted in the test. The temperature was maintained at 42°C and reactions routinely monitored by measurements of total solubilised iron, pH and redox potential (Fe^{3+}/Fe^{2+}). Other analytical measurements are described below.

Experiments involving the study of the toxicities of As(III) and As(V) were carried out in shaken flasks, using pyrite as a substrate,usually at 1% pulp density. The As(V) was added as sodium arsenate, while As(III) stock solutions were prepared by dissolving As_2O_3 in sodium hydroxide solution and acidifying to pH 2.10 with concentrated sulphuric acid. Experiments were initiated by adding a suspension of cells previously grown on Fe(II) as a substrate. Growth was followed by monitoring the solubilization of iron.

Mineral Substrates

The arsenopyrite concentrate contained 32.4% FeSAs and 30.9% FeS_2. The sample used was ground to -45 μm. Pyrite samples were ground to -38 μm.

Analytical Methods

Samples of the supernatant solution were acidified further with concentrated hydrochloric acid and analyzed for total iron and total arsenic by atomic absorption spectroscopy, using a Perkin-Elmer 2380 instrument. Concentrations of arsenic(III) were determined polarographically using an EG&G model 264A polarograph and EG&G model 303A dropping mercury electrode. Concentrations of iron(II) were determined calorimetrically using 1,10-phenanthroline. Optical densities were measured on a Pye-Unicam SP6-550 spectrophotometer.

RESULTS

Toxicity of As(III) and As(V) to the Mixed Culture

The effects of As(III) and As(V) on growth of the mixed culture (growing on pyrite as substrate and unconditioned to high arsenic levels) are shown in Figures 1 and 2 respectively. Generally, increase in concentration of the arsenic compound resulted in a decrease in the amount of iron solubilised. The effect of As(III) was more marked than that of As(V), being approximately two to three times more toxic. However, as will be shown, some oxidation of As(III) to As(V) occured during these experiments. Accordingly, particularly at low concentrations, the concentration of As(III) may have been substantially diminished in the later stages of the experiments shown in Figure 1. This suggests that the toxicity of As(III) may have been underestimated in these experiments.

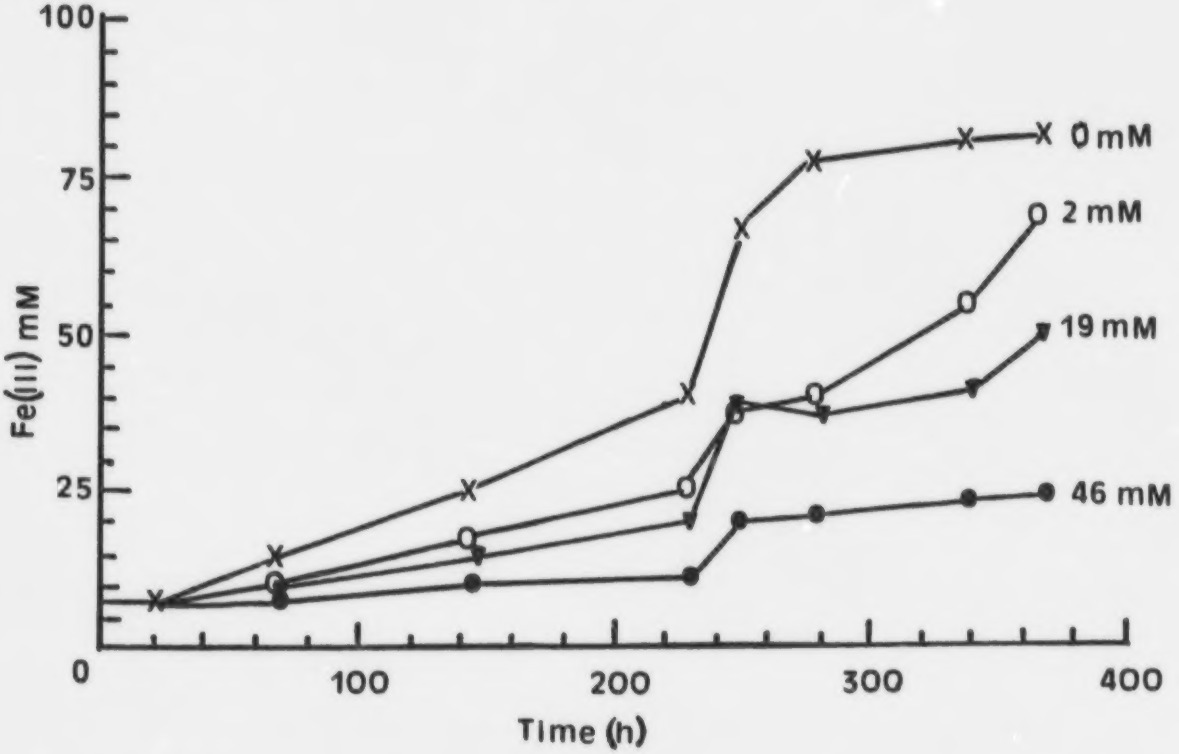

Fig. 1 Effect of As(III) upon Growth of Culture

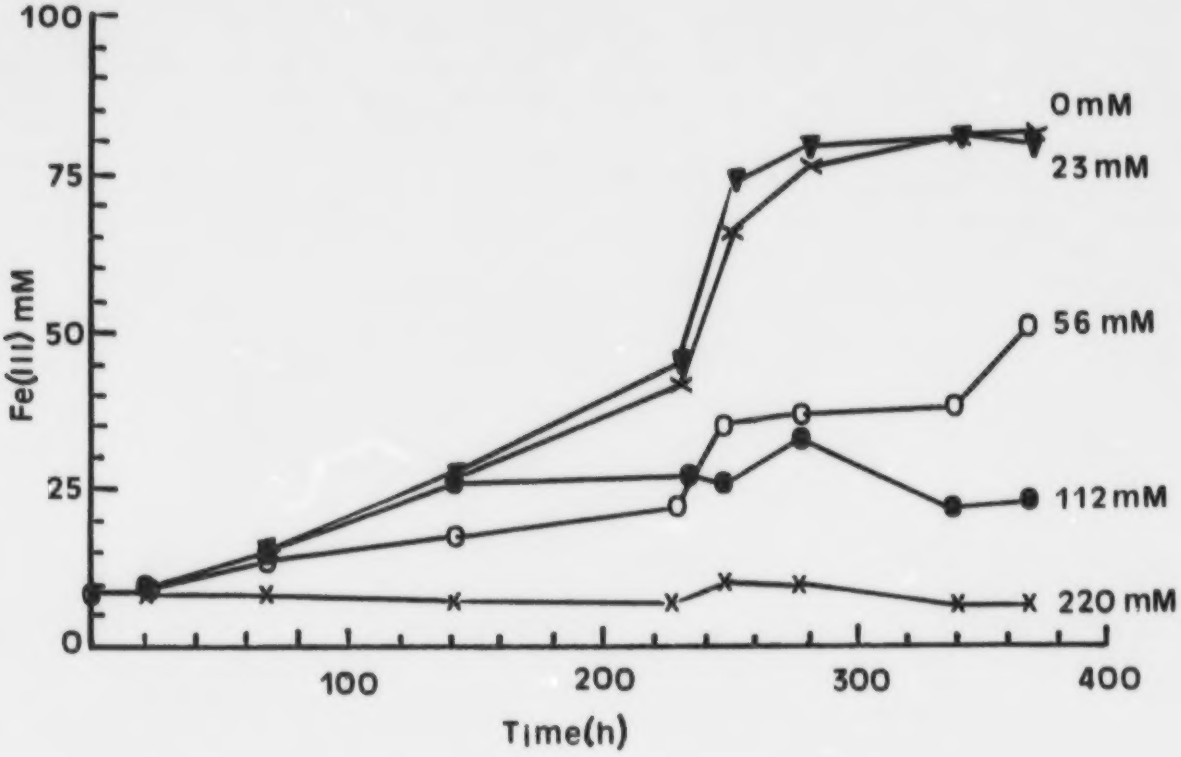

Fig. 2 Effect of As(V) upon Growth of Culture

Oxidation of Arsenic(III)

Inoculation of medium containing pyrite and arsenic(III) with the mixed culture results in a long lag phase (Figure 1). We have shown that the concentration of As(III) in the medium gradually decreased during this period. The rate of loss of As(III) in the medium is shown in Figures 3 [2.5 mM as(III)] and 4 [7.0 mM As(III)] and increases substantially after a period of 50-60 hours. A comparison of these figures with Figure 1 shows that some Fe(III) was produced from the mineral in the time period when the concentration of As(III) was decreasing. The availability of Fe(III) is compatible with the suggestion that the loss of As(III) was due to oxidation by Fe(III) to give the less toxic As(V). A comparison of redox potentials for Fe(III) and As(V) at pH 1.5 in sulphate medium suggests that Fe(III) should be able to oxidize As(III) under these conditions. Values of E are: Fe(III)/Fe(II), 0.67V; As(V)/As(III), 0.47V; S(VI)/S(O), 0.28V. However, while Fe(III) should readily oxidize S(O), the difference in potential between the Fe(III)/Fe(II) and As(V)/As(III) couples is small and it is difficult to predict whether Fe(III) will oxidize As(III) under the conditions used in these experiments. We have therefore attempted to study the reaction between As(III) and Fe(III) over a wide range of conditions at temperatures up to 70°C, and have found no evidence for it taking place over the time period relevant to the present work. It appears, therefore, that the conversion of As(III) to As(V) in the bioreactor does not involve direct oxidation by Fe(III) probably due to a kinetic restriction. It should be noted that these results do not exclude the possibility that the Fe(III)-As(III) reaction could occur if an appropriate catalyst is present.

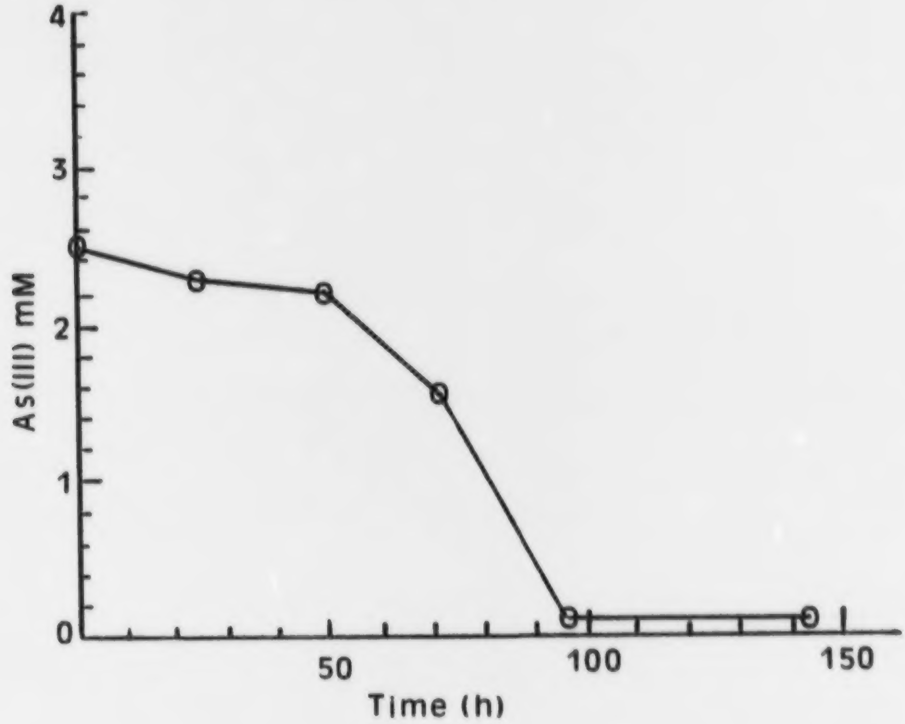

Fig. 3 Oxidation of As(III) (2.5 mM) during lag phase

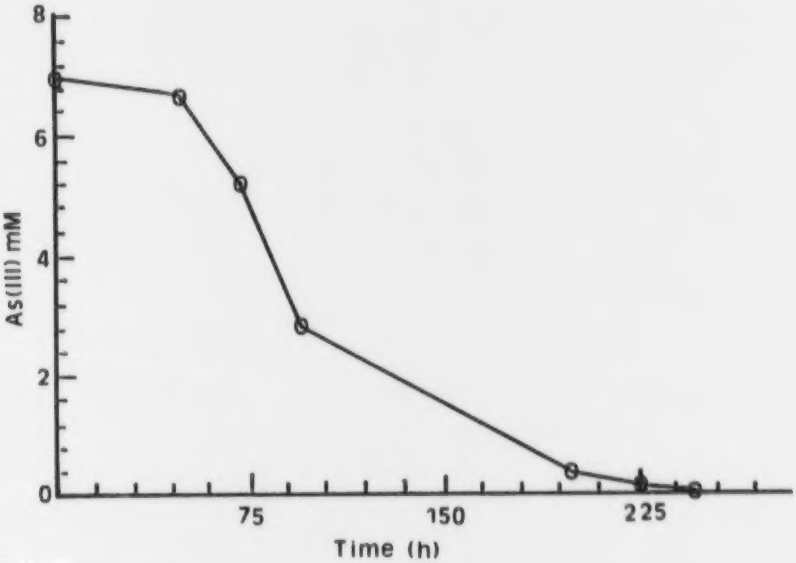

Fig. 4 Oxidation of As(III) (7 mM) during lag phase

In view of the lack of evidence for a chemical pathway for the oxidation of As(III), the possibility of a biological oxidation pathway was then considered. A culture of Fe(II)-grown cells were centrifuged and the pellet taken through several cycles of washing and centrifugation to remove Fe(III) as far as possible. These cells were then added to weak medium containing appropriate concentrations of As(III). However, oxidation of the As(III) did not occur over an extended time period, demonstrating that direct oxidation by cells is unlikely to account for the formation of As(V) in the supernatant solution. A parallel experiment with another sample of the same batch of washed cells demonstrated that they were able to oxidize pyrite and were therefore viable. These experiments also show that the moderately thermophilic culture cannot derive the energy for chemoautotrophic growth from the oxidation of As(III).

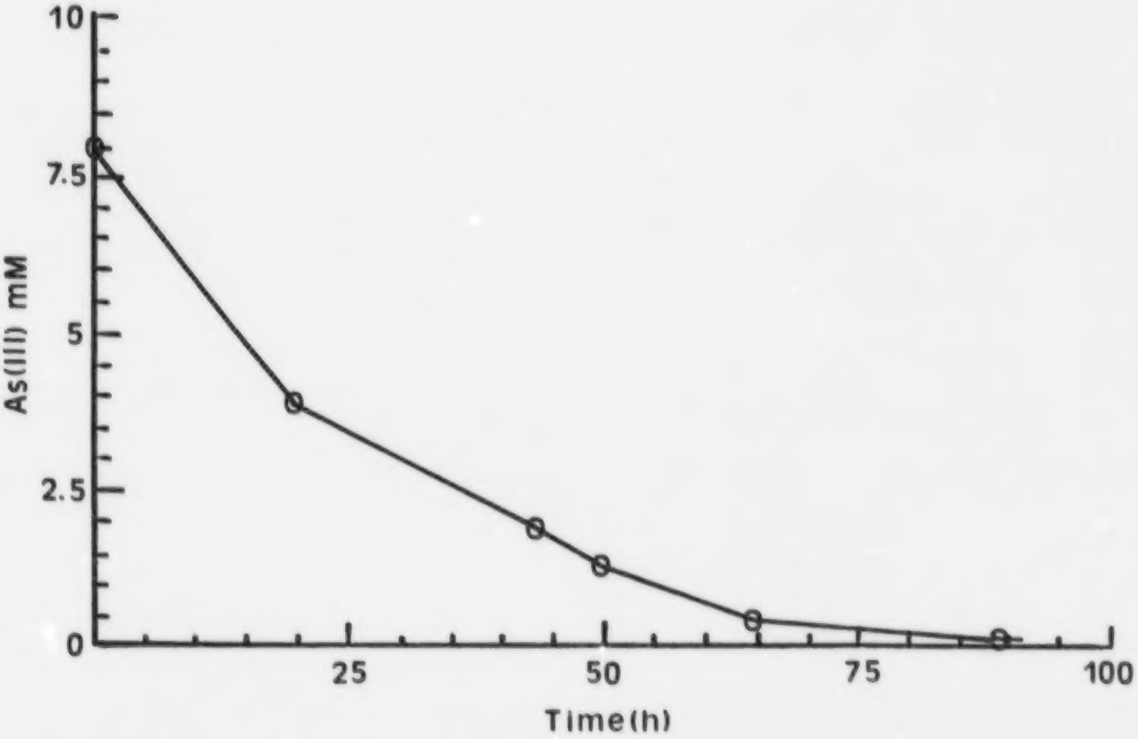

Fig. 5 Oxidation of As(III) by Fe(III) plus Mixed Culture

However, in contrast, a combination of mixed culture (growing on pyrite) and Fe(III) was able to oxidize As(III) completely to As(V), as shown in Figure 5. One explanation for this result could have been that the Fe(III) was complexed to a ligand released by the cell or to the cell surface, and that the redox potential of the resulting complex was sufficiently enhanced to allow the oxidation of As(III) to occur. Accordingly, an experiment was carried out with dead cells plus Fe(III). This combination was unable to oxidise As(III). It is difficult to draw confident conclusions from this type of experiment, as the Fe(III) may be complexed to a ligand released as a result of the metabolism of the cell, but, on the basis of the present evidence, it appears probable that the cells play some direct role in the oxidation of As(III), provided that Fe(III) is also present. Evidence has been produced for the ability of an acidophilic organism isolated from the acidic drainage water from a pyrite mine to oxidise As(III) over 1-1.5 h under certain circumstances (Wakao *et al.*, 1988). As(III) in a water sample was oxidized almost completely in 5 days when incubated at 30°C. Oxidation did not take place at 4°C, confirming bacterial action. However, these bacteria differ from the present culture in that they were unable to oxidise Fe(II) and elemental sulphur as energy sources. These authors established that *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* were unable to carry out this reaction.

Formation of Arsenic(III)

Analysis of supernatant solutions from bio-oxidation reactors showing toxic effects from arsenic gave As(III) concentrations as high as 30-40 mM (2.25-3.0g L^{-1} As), while in dead cultures concentrations were as high as 70-90 mM (5.25-6.75g L^{-1} As). We investigated the possibility that the production of As(III) involves a chemical route. Figure 6 shows the release of As(III) with time when the arsenopyrite concentrate (at 5% pulp density) was treated with the standard medium at pH 1.5. and the much greater effect when the concentrate was treated with a synthetic bio-oxidation solution. A limited amount of As(III) was released by the action of the medium, the concentration reaching a maximum value after about 10 minutes. Some As(V) was also produced in solution (results not shown). These results may well have been due to the solubilization by the acidic medium of the products of surface oxidation of the arsenopyrite during the milling process. In contrast, the effect of the bio-oxidation solution is more substantial. There is a rapid release of As(III) over the first five minutes and a subsequent slower release that levelled off after two days. The total amount of As(III) released amounted to about 16% of the total arsenic present in the concentrate used in the experiment. It should be noted that the initial maximum rate of release of As(III) by this route is less than half of the overall rate of bio-oxidation of arsenopyrite at 5% solids.

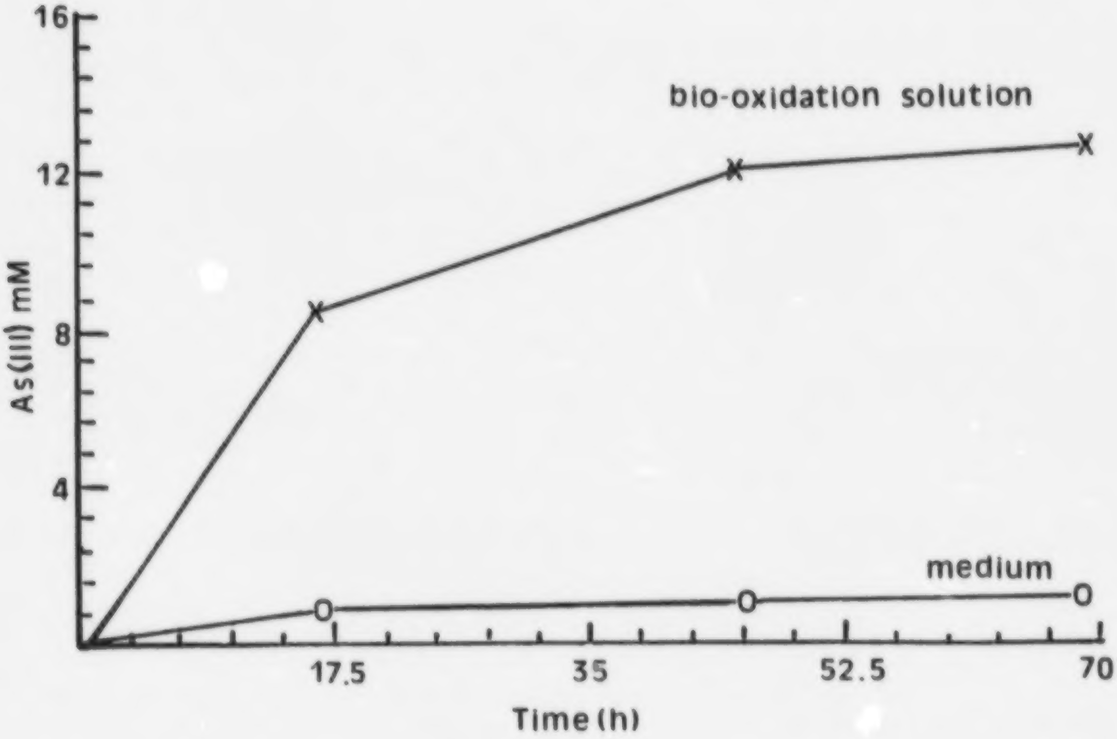

Fig. 6 Release of As(III) from arsenopyrite concentrate (at 5% solids) by Medium and by Synthetic Bio-oxidation Solution

CONCLUSIONS

The scale on which As(III) was produced during the bio-oxidation of arsenopyrite must mean that it was associated with a major reaction pathway in the biological oxidation of arsenopyrite. The concentrations detected during bio-oxidation considerably exceeded those formed by chemical reactions involving arsenopyrite. The concentrations detected during bio-oxidation considerably exceed those formed by chemical reactions involving arsenopyrite. The extent to which As(III) was detected in solution during bio-oxidation reflects the balance between the rate of production of As(III) from arsenopyrite and the rate of oxidation of As(III) to As(V). Inhibition of the second step will result in the accumulation of As(III) and the aggravation of toxic effects. It is clearly important that the concentrations of As(III) in reactors be monitored on a regular basis.

The oxidation of As(III) to As(V) appears to involve a well-defined role for the bacteria in association with Fe(III). No evidence for direct oxidation of As(III) by Fe(III) was founded. The possibility that the final stage of the oxidation of arsenic in arsenopyrite to give As(V) occurred in the medium rather than on the mineral surface has the interesting implication of requiring bacteria to be present in the supernatant solution.

ACKNOWLEDGEMENTS

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TOXICITY OF ARSENIC COMPOUNDS TO THE SULPHUR-DEPENDENT ARCHAEBACTERIUM SULFOLOBUS

by

E. Börje Lindström and H. Mikael Sehlin**

ABSTRACT

Toxicity studies of arsenate ($AsO2_4^{3-}$) and arsenite (AsO_2-) ions were performed with *Sulfolbus acidocaldarious* "strain BC" at 70°C. The toxic level of arsenate was between 10 and 20 mM. The arsenite ion showed a primary effect on the growth rate of *Sulfolbus*, and that detoxification was due to oxidation to arsenate. This activity was shown to persist in a cell-free extract of *Sulfolbus*.

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* Unit for Applied Cell and Molecular Biology, University of Umeå, S-901 87 UMEÅ, Sweden

TOXICITÉ DES COMPOSÉS DE L'ARSENIC SUR *ACHAEBACTERIUM SULFOLOBUS*, UNE ESPÈCE BACTÉRIENNE DÉPENDANTE DU SOUFRE

E.B. Lindstrom et H.M. Sehlin

RÉSUMÉ

Des études de toxicité des ions arséniate (AsO_4^{3-}) et arsénite (AsO_2-) ont été menées sur la souche "BC" de *Sulfolbus acidocaldarius* à 70°C. Le niveau toxique de l'arséniate se situait entre 10 et 20 mM. Ces études ont démontré que l'ion arsénite avait une incidence importante sur le taux de croissance de *Sulfolbus* et que la détoxification était attribuable à l'oxidation de l'arséniate. Il a aussi été démontré que cette activité persistait dans un extrait de *Sulfolbus* acellulaire.

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* Unité de Biologie Moléculaire et Cellulaire Appliquée Université de Umeå, UMEÅ, Sweden

INTRODUCTION

The bacteria used in biohydrometallurgical processes are often exposed to solutions with very high metal concentrations. Several of these metal ions are extremely toxic to the bacteria and therefore interfere with the rates of the leaching process.

Arsenic is solubilized when e.g., gold containing arsenopyrites are bioleached. It has been suggested that arsenic is released as arsenite and chemically oxidized to arsenate by oxygen (8).

The toxicity of arsenic ions and the resistance mechanisms to these in bacteria has so far been studied only in eubacteria. In several of these bacteria the genes mediating the resistance to arsenate have been located on plasmids. The resistance mechanism to arsenate is dependent on the phosphate uptake mechanism (6,13). Very little is, however, known about the toxicity of and resistance to the arsenite compound, although it has been reported to be much more toxic than the arsenate (12). Osborne and Ehrlich (7) have shown that the enzyme arsenite oxidoreductase is detoxifying the compound and Abdrashitova *et al.* (1) suggest that formation of peroxide is responsible for the detoxification. A plasmid-coded arsenite pump (10) is present in *Escherichia coli*.

The knowledge about the resistance to arsenite and arsenate Archaebacteria is very limited. This paper shows the toxic level of arsenate for *Sulfolobus* and that this archaebacterium can oxidize arsenite and arsenate.

MATERIAL AND METHODS

Organism and Growth Conditions

Sulfolbus acidocaldarius "strain BC" (5) was used throughout this investigation. The mineral salt solution 9K (without ferrous iron) (11), was used and adjusted to pH 2.0 with sulphuric acid. Potassium tetrathionate (Merck), pyrite (FeS_2) or arsenopyrite (FeAsS), were used as energy sources.

Mineral

The minerals were kindly provided by Boliden Mineral AB, Sweden and were flotation concentrates with particles size less than 0.5 mm. Pyrite contained 38.5% (w/w) of iron and 51.8% (w/w) sulphur. The main chemical composition of arsenopyrite (Olympus) was: iron 38.7% (w/w), sulphur 40.5% (w/w) and arsenic 11.3% (w/w).

Resistance to Arsenate and Arsenite

The experiments were performed in 250 mL conical flasks with 100 mL of 9K mineral salts medium, supplemented with 5 mM potassium tetrathionate. Various concentrations of arsenite or arsenate were added at the start of the experiments, and a 5% (v/v) of a log-phase culture of *Sulfolbus* was used as inoculum. The flasks were shaken at 130 rpm at 64°C.

Growth was followed by measuring optical density with 1 cm cuvettes at 440 nm with a Hitachi spectrophotometer model 150-20.

Induction of Arsenite Oxidation

The experiments were carried out in glass fermentors. *Sulfolbus* was grown in a total volume of 500 mL containing 10 mM potassium tetrathionate. Arsenite was added to 1 mM final concentration, when optical density at 440 nm had reached approximately 0.1, or the growth had reached the stationary phase. Growth and arsenate formation were followed daily.

Preparation of Cell-Free Extract

Bacteria were grown in Nalgene plastic flasks in 700 mL 9K-medium containing 10 mM potassium tetrathionate, 0.1% casamino acids, 1 mM NaCl and, in one of the flasks, 2.5 mM arsenite. The bacteria were harvested by centrifugation in a JA10-rotor (Beckman) for 15 minutes at 8000 x g. After resuspension in 9K-medium, the bacteria were centrifuged in a JA20-rotor (Beckman) for 5 minutes at 12000 x g. The bacteria were then suspended in 1 mL of 9K-medium and sonicated on ice with a Branson Sonifier Cell Disrupter B15, 4 x 30 seconds at range 5, using the microtip. Cooling was allowed for 30 seconds in between the sonications. After the sonication, the extracts were centrifugated for 10 minutes in an Eppendorf centrifuge at maximum speed.

Arsenite Oxidation With Cell-Free Extract

The reaction mixtures contained 100 ul of the cell-free extract, 800 ul 9K-medium, pH 2.0, and 100 ul 100 mM arsenite. After mixing the incubation was performed at 70°C. Samples were withdrawn after 0, 0.5, 1, 2, 4, and 24 hours, diluted 200-fold and analyzed for arsenate content.

Analysis of Arsenate, Arsenite, and Tetrathionate

Arsenate was determined by ionic chromatography (LDC/Milton Roy) with the elution buffer 3.0 mM $NaHCO_3$-2.0 mM Na_2CO_3, pH 10 and using the cationic pre-column Dionex HP1C AG4A and the column Dionex HP1C AS4A. The flowrate used was 2 mL/min.

During growth with tetrathionate as energy source samples were withdrawn from the cultures and diluted 100 to 200-fold in the elution buffer. The samples were filtered through a 0.2 μm filter (Sartorius Minisart N) before chromatographed.

When pyrite/arsenopyrite were used as energy sources the iron must be removed before the chromatography step. The samples were therefore digested in hydrochloric acid (1:4 dilution in 5M HCl) for 30 minutes at 65-70°C, and usually 0.5 mL aliquots of digested samples were then applied to a catonic column containing 2 mL hydrogen form resin 50W-X8, 100-200 Mesh, Bio-Rad (3,4). The eluate was collected and adjusted to 10 mL and divided into two parts, of which one was oxidized with aqua regia according to Liang and Dutrizac (3,4) and arsenite was calculated as the difference in the arsenate content of these two parts. To avoid overloading of the anionic column the chloride was removed by evaporation to dryness followed by resuspension in the same volume of the elution buffer used in the ionic chromatography step (see above).

The amount of tetrathionate in the samples was determined by the method of Kelly *et al.* (2) adapted to a Technicon Autoanalyzer. Potassium thiocyanate, 0-10 mM, in 9K-medium was used for the standard curve.

RESULTS

Release of Arsenic During Bioleaching

In thermophilic bacterial leaching of the mineral arsenopyrite (FeAsS), we have observed that arsenic was released from the mineral initially as arsenite. At the end of the leaching experiment only arsenate was detected. This was confirmed in the experiment shown in Figure 1, where the leaching was started on 1% (w/v) pyrite and 1.5% (w/v) arsenopyrite was added as indicated.

Resistance to Arsenate and Arsenite

The toxic levels of arsenate and arsenite were investigated for *Sulfolbus* during growth in liquid media. In presence of arsenate, 0 to 10 mM, the growth rates are not influenced. Figure 2 shows that the toxicity level of arsenate for *Sulfolbus* is between 10 and 20 mM. When arsenite was used in the same type of experiment, this compound markedly influenced, contrary to arsenate, the growth curve. The toxicity of arsenite was tested in the range of 0 to 10 mM. At 1 mM arsenite, growth was already significantly retarded (Figure 3). In the case of both 5 and 10 mM there was even a lag-period of about 100 hrs. before the growth started and then with almost the same growth rate as the control. The maximum yields were nearly also the same for these cultures. Thus arsenate influences the maximum growth capacity, while the effect of arsenite was primarily on the growth rate.

Arsenite Oxidation During Bacterial Growth

In order to study the kinetics of arsenic transformation by *Sulfolbus*, arsenate was monitored as a function of growth phases for the arsenite cultures. In Figure 4 the result with the 5 mM arsenite culture is shown. Arsenate was not detected until in late exponential growth phase of this culture. The formation of arsenate is strongly correlated to the growth of the bacterium. It seems to be a linear rather than an exponential function of time. This continues even a couple of days after the decline of the growth. Then the formation of arsenate leveled off slightly.

Induction of Arsenite Oxidation

The induction of arsenite oxidation during growth of *Sulfolbus* on 10 mM tetrathionate was studied in the experiment shown in Figure 5A and B. The growth rate was reduced from 0.077 to 0.048 doublings per hour, when the arsenite was added. Arsenate appeared after 40 hours when the arsenite was added during the exponential growth phase, (Figure 5A). No arsenate was found when the arsenite was added in the stationary phase (Figure 5B).

Thus the induction of arsenite oxidation in *Sulfolbus* is dependent on actively growing cells.

Arsenite Oxidation by Cell-Free Extracts

As the first step in the purification of the enzyme(s) responsible for arsenite oxidation in *Sulfolbus* a cell-free extract was prepared from a culture grown on 10 mM tetrathionate and which had been induced for arsenic oxidation in the exponential growth phase. Table 1 shows that arsenite oxidation can take place without living cells, but that most of the activity still is associated with the cell debris.

DISCUSSION

Resistance level of bacteria to antibiotics, metals or other compounds can be determined either in liquid or on solid media. The lethal dose for 50% survival (LD^{50}) is often used. Normally the liquid cultures are inoculated and when growth has ceased, the OD-value obtained is plotted against the concentration of the toxic compounds. We have used this method in this study, but due to the decline in OD(440 nm), when the energy source has been used up, we followed the growth and recorded the optimum OD value.

When arsenite was used we confirmed what has been reported for eubacteria that this compound was more toxic to the bacteria than arsenate (12). The growth rate was effected already at 1 mM arsenite and for higher arsenite concentrations, 5-10 mM, the growth was completely halted for a while. However, after varying lag-periods the growth resumed with almost the same growth rate as the control culture without any added arsenite, and the yield was also in the same range. In these cases we found that the arsenate, and the yield was also in the same range. In these cases we found that the arsenite had been oxidized and therefore the toxic level of the metal had increased to that of arsenate. The oxidation of arsenite seems to occur only in late exponential growth phase which is in agreement with what has been reported for *Alcaligenes faecalis* (7,9). How the arsenite is acting on *Sulfolbus*, we don't know yet. We have tried to analyze the growth medium after the addition of arsenite and found very little oxidizable free arsenite in the medium. Our preliminary data suggest that the arsenite is bound to the cells and is released as arsenate when the bacteria lyses at the end of the exponential phase (data not shown).

Our induction experiments also show varying elapsed time before the oxidized product appears. It seems that the oxidizing mechanism is connected in some way to the energy metabolism, in our case tetrathionate. When the energy source is almost used up, the arsenate product does appear. If this is due to lysis of the bacteria or due to induction of an enzyme in late exponential growth phase is not known. No induction occurs when arsenite was added in the stationary phase.

As we have developed an *in vitro* assay for the arsenite oxidation and that the cell-free extract has shown enzymatic activity, we hope to be able to purify the responsible enzyme/enzymes and be able to study the mechanism in more detail.

CONCLUSIONS

1. The toxic level of arsenate in defined liquid medium was between 10 and 20 mM for *Sulfolbus acidocaldarius* "strain BC".
2. Arsenite is detoxified through biological oxidation to arsenate with this *Sulfolbus* strain.
3. A cell-free extract was shown to catalyze oxidation of arsenite to arsenate.

ACKNOWLEDGEMENTS

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TABLE 1

Arsenic oxidation with cell-free extract

Sample	Arsenate formation (nmoles in 24 hrs)
9K medium	160
Supernatant	2270
Resuspended pellet	5718
Control	360

Harvested, washed cells, grown in presence of arsenite, were sonicated for 4 x 15 seconds. The extract was centrifuged for 10 minutes in an Eppendorf centrifuge (full speed), the supernatant was collected and used as cell-free extract. The pellet was then resuspended to the same volume in the mineral medium 9K, pH 2. Cell-free extract made from *Sulfolbus* grown in the absence of arsenite, was used as the control sample. All reaction mixtures were run according to Material and Methods.

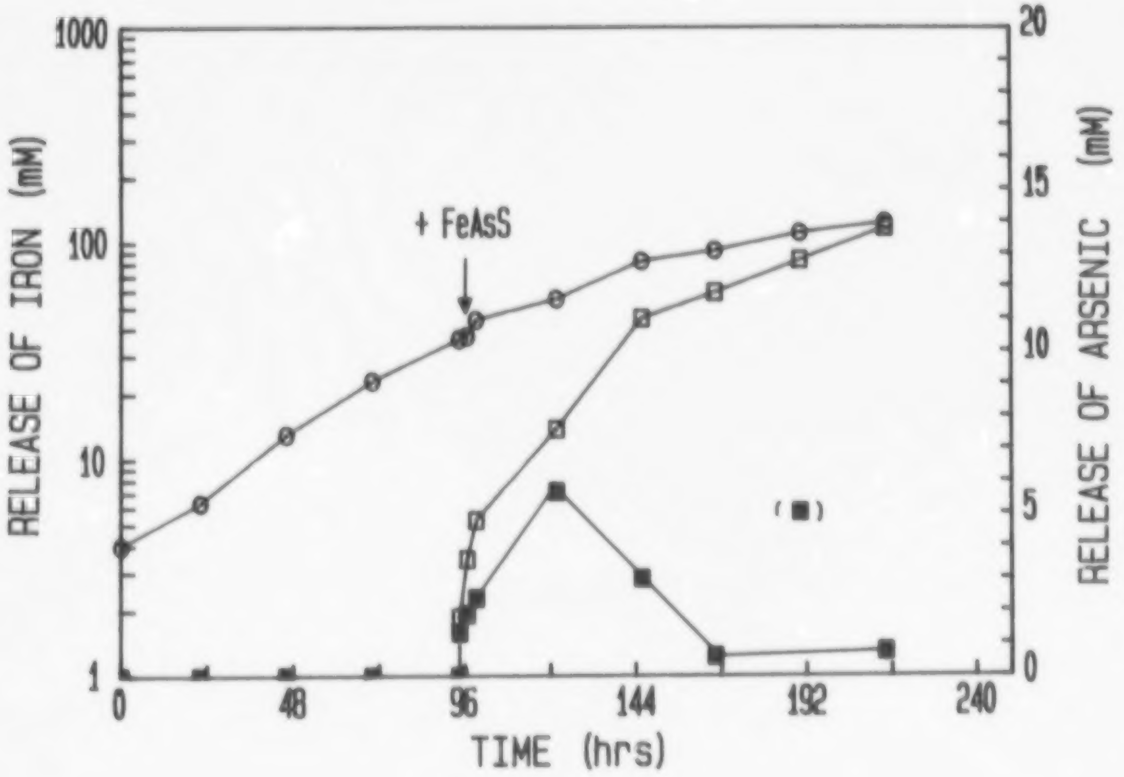

Fig. 1 Release of arsenic during bioleaching. The arrow shows the addition of 1.5% (w/v) of arsenopyrite (Olympus) during leaching of 1% (w/v) pyrite with *Sulfolbus*. The release of total arsenic, (□), was monitored with atomic adsorption spectroscopy and the arsenate by ionic chromatography according to Material and Methods. The arsenite, (■), was calculated as the difference. (○), release of total soluble iron.

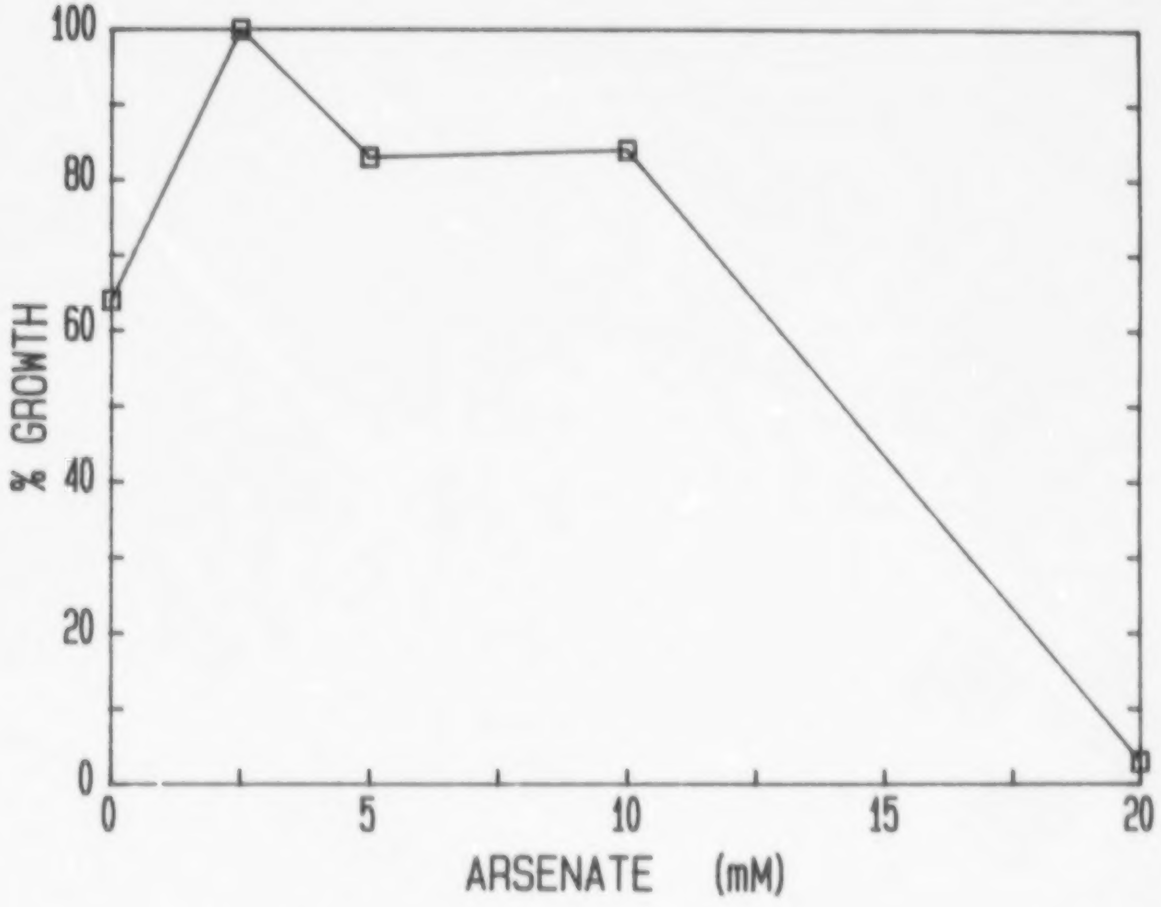

Fig. 2 Resistance of *Sulfolbus* to arsenate. Five 250 mL conical flasks containing 100 mL 9K-medium and 5 mM tetrathionate were supplemented with 0,2.5, 5,10, and 20 mM arsenate respectively. After inoculation with *Sulfolbus*, the growth was monitored by optical density at 440 nm. The maximal OD(440nm)-value of these cultures were used as 100%.

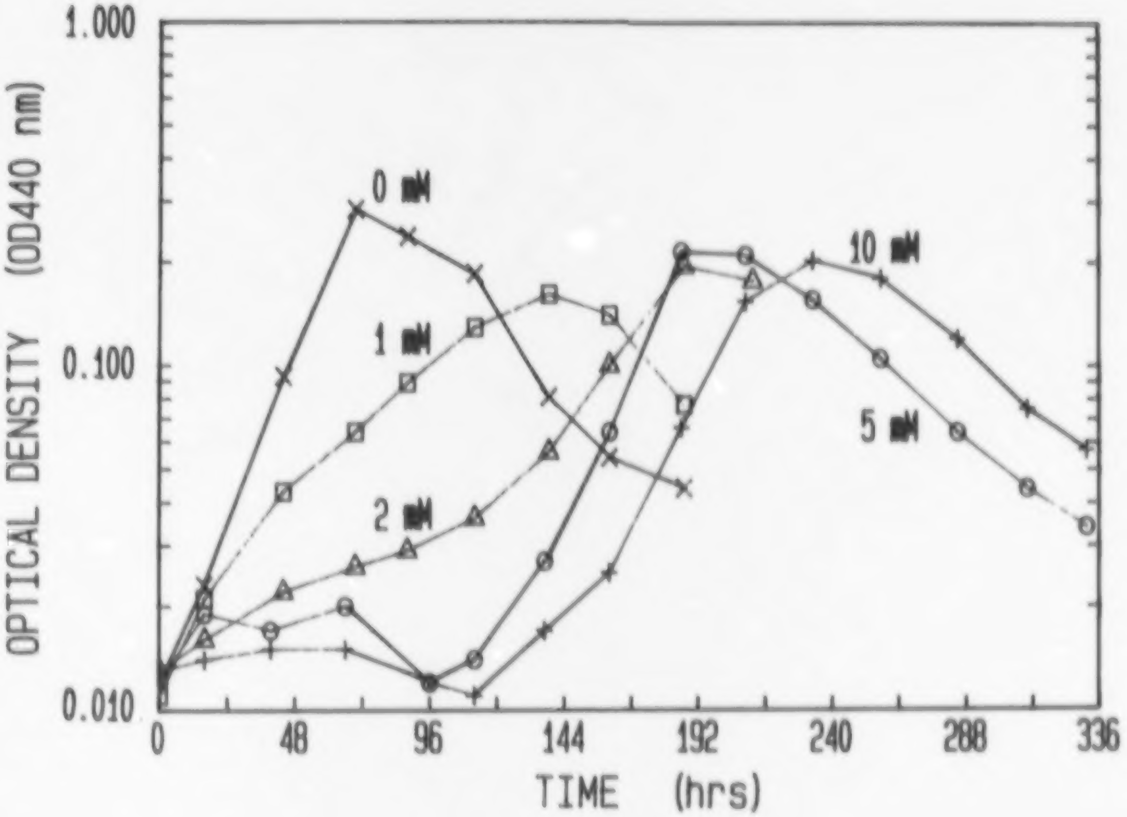

Fig. 3 The effect of arsenite on the growth of *Sulfolbus*. Five 250 mL conical flasks containing 100 mL 9K- medium and 5 mM tetrathionate were supplemented with 0, 1, 2, 5, and 10 mM arsenite. After inoculation with *Sulfolbus* the growth was monitored by optical density at 440 nm. (X), 0 mN; (□), 1 mM; (Δ), 2 mM; (○), 5 mM; and (+), 10 mM arsenite.

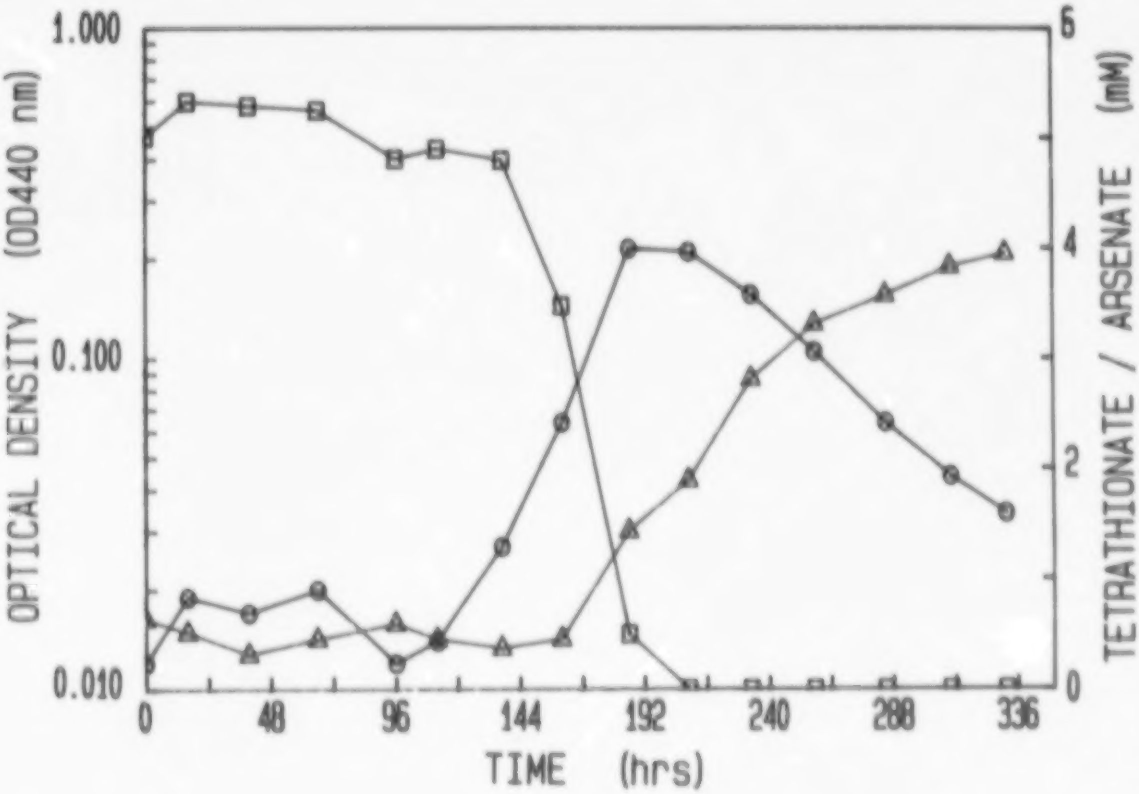

Fig. 4 Arsenite oxidation during bacterial growth. *Sulfolbus* was grown in liquid 9K-medium supplemented with 5 mM tetrathionate and 5 mM arsenite. At indicated times samples were withdrawn for optical density, OD440nm), (○), measurements and for analysis of tetrathionate, (□), and arsenate, As(V), (△).

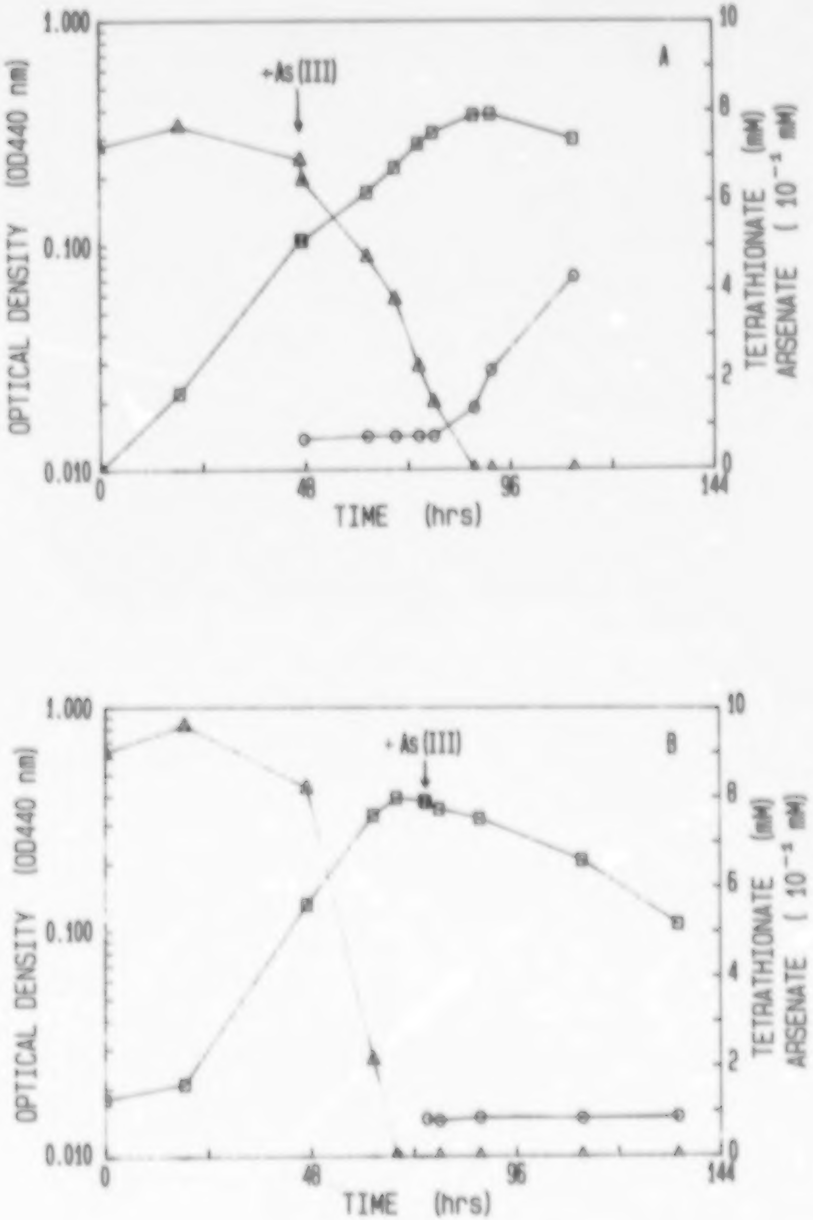

Fig. 5 Induction of arsenite oxidation in *Sulfolbus*. *Sulfolbus* was grown in 9K-medium supplemented with 10 mM tetrathionate and growth was monitored as optical density at 440 nm, (□).
Arrows indicate the additional of 1 mM arsenite:
A) in exponential growth phase
B) in stationary growth phase
Samples were withdrawn at indicated times and analyzed for tetrathionate, (△), and arsenate (○).

DEVELOPMENT OF ORE BIOLEACHING STANDARDS

G.J. Olson

Polymer Division, National Institute of Standards and Technology,
Gaithersburg, MD 20899, USA

ABSTRACT

Discussion at past bioleaching conferences has indicated the need for standard procedures and test materials for improved intercomparison of data from pyrite and ore bioleaching tests. Many variables are associated with bioleaching rates and the availability of standards would provide a means for comparison of strains of microorganisms and ore substrates. Pyrite from South Carolina was tested as an initial candidate reference material in an earlier study. Three strains of *Thiobacillus ferrooxidans* were found to leach a 100-200 mesh fraction of the material in shake flasks at rates ranging from 1.7-2.2 mg Fe/l/hr with relative standard deviations of 7-11%. Inoculum size did not affect bioleaching rates at initial cell densities of 5×10^6 or higher. Baffled flasks did not increase pyrite oxidation rates. These and other data were incorporated into a pyrite leaching procedure. Recent efforts focused on pyrite obtained in bulk from the Waldo Mine in New Mexico. The leaching rate of this pyrite under varying conditions ranged from 3.08 to 13.2 mg Fe/l/hr with a strain of *T. ferrooxidans* (American Type Culture Collection no. 13661). The Waldo pyrite is being characterized for composition and will be available for distribution from the Office of Standard Reference Materials, National Institute of Standards and Technology.

ÉLABORATION DE NORMES CONCERNANT LA BIOLIXIVIATION DU MINERAI

G.J. Olson

*Polymer Division, Institut National des Standards
et de la Technologie, Gaithersburg, MD É.-U.*

RÉSUMÉ

Des discussions au cours de conférences sur la biolixiviation tenues par le passé ont démontré la nécessité de développer des procédures et des matériaux d'essai standard pour améliorer la comparaison des données recueillies au cours d'essais de biolixiviation du minerai et de la pyrite. De nombreuses variables ont une incidence sur les taux de lixiviation et des normes permettraient de comparer les sources de micro-organismes ainsi que les minerais. La pyrite de la Caroline du Sud a été le premier matériau auquel on a fait subir des essais en vue de l'utiliser comme matériau de référence. On a découvert que trois souches de *Thiobacillus ferrooxidans* pouvaient lixivier des fragments de maille de 100 — 200 dans des fioles à des taux variant de 1,7 à 2,2 mg Fe/L/h, les écarts standards variant de 7 à 11 pour cent. La taille de l'inoculum n'a pas eu d'incidence sur les taux de biolixiviation à des densités cellulaires d'au moins 5 x 10^n. Les taux d'oxydation de la pyrite n'ont pas été plus élévés dans les flacons obturés. Ces données ainsi que d'autres ont été incorporées à une procédure de lixiviation de la pyrite. Récemment, des efforts ont porté sur la pyrite en vrac de Waldo Mine au Nouveau-Mexique. Le taux de lixiviation de cette pyrite sous différentes conditions par une souche de *T. ferrooxidans* (référence américaine de culture type n° 13661) a varié de 3.1 à 13 mg Fe/L/h. Des travaux portant sur la caractérisation de la composition de la pyrite de Waldo Mine sont en cours et celle-ci pourra bientôt être obtenue de l'Office of Standard Reference Materials, National Institute of Standards and Technology.

INTRODUCTION

Recent years have seen a rapidly growing interest in research and applications involving microorganisms for processing of metal sulfide ores and desulfurization of coal. However, intercomparison of bioleaching rate data has been difficult, hindering assessments of the effectiveness of different microorganisms or the susceptibility of certain ore deposits to microbial attack. The availability of standard ore substrates and procedures for conducting bioleaching tests would facilitate data intercomparison by researchers, including the identification of superior strains and assessment of ore deposits for bioleaching. Energy, Mines and Resources Canada produces mineral certified reference materials including a number of important Canadian oxide and sulfide ores (Steger, 1986). The National Institute of Standards and Technology (NIST) Office of Standard Reference Materials also offers a variety of mineral standard reference materials but no sulfide ores (Seward, 1988). However, despite the importance of pyrite in metal bioleaching and coal biodesulfurization, a pyrite reference material has not been available. This paper describes the preparation and testing of a pyrite research material at NIST. Factors affecting the bioleaching rate of this material are also described. The method for determination of bioleaching rates presented in this paper forms the basis for a proposed American Society of Testing and Materials (ASTM) procedure for conducting pyrite bioleaching rate determinations.

MATERIALS AND METHODS

Processing of Pyrite

Approximately 20 kg of -10 mesh pyrite from the Waldo Mine in New Mexico was obtained from G. Griswold, New Mexico Tech, Socorro, NM. Preliminary tests showed that CO_2 was evolved (as determined by mass spectrometry) upon exposure of the pyrite to dilute HCl, indicating the presence of acid-neutralizing carbonates in the material. Therefore, the material was washed by mixing with 4.6M HCl for 30 min, rinsing several times with deoxygenated deionized water, and drying under a flow of nitrogen at 60°C. The above process was repeated since the once-washed material still showed gas evolution, albeit more slowly, upon exposure to dilute HCl. After the second wash, little if any bubbling of the material was noted in the presence of HCl. The washed and dried material was ground for 1-2 hours in a ceramic ball mill, and about 13 kg of -165 +250 mesh fraction (58-91 μm was obtained. After thorough mixing, the pyrite was dispensed in 100 g portions into 127 glass bottles. The bottles (fitted with aluminum foil covers) were heated at 125)°C in a vacuum oven for 1.5 hr. After cooling to room temperature, plastic screw caps (sterilized by autoclaving) fitted with liners (soaked in 95% ethanol, dried at 37°C) were placed loosely on the glass bottles containing the pyrite. The bottles were again placed in the vacuum oven which was evacuated at room temperature. The atmosphere was replaced with high purity nitrogen. The caps were tightened and the bottles were vacuum-sealed in foil pouches. Seven bottles were retained for analyses and 120 bottles placed in inventory with the National Institute of Standards and Technology Office of Standard Reference Materials. The material is identified as "pyrite research material".

Chemical Determinations

X-ray diffraction. X-ray powder diffraction patterns were collected using a Phillips diffractometer. Pyrite samples from two different bottles were ground in an agate mortar and were analyzed at 0.1° intervals (for 1.0 sec) from 5-70°.

Scanning electron microscopy-energy dispersive x-ray microanalysis (SEM-EDX). Pyrite powder was fixed to aluminum stubs with low resistance contact cement (Ernest F. Fullam, Inc., Latham, NY) and examined at an accelerating voltage of 20 kV with a scanning electron microscope (Amray 1000) equipped for energy dispersive x-ray microanalysis (EG&G Ortec System).

Fourier transform infrared spectroscopy (FT-IR). An admixture of pyrite (8-12 mg) and KBr (500 mg, International Crystal Laboratories, Garfield, NJ) was ground for 10 sec in a Wig-L-Bug mixer (Crescent

Dental Implement Co., Lyons, IL). The diluted pyrite was analyzed using a Fourier transform infrared spectrometer equipped for diffuse reflectance operation (Analect Instruments, Utica, NY).

Microbiological Studies

Sterility testing. Approximately 0.2 g of pyrite from each of six bottles was added to 10 ml of several media in 18 x 120 mm test tubes: 1) 9K medium salts (Silverman and Lundgren, 1959) containing ferrous sulfate (1.47% w/v, pH 2.5), elemental sulfur (0.1 g/tube, pH 3.8) or sodium thiosulfate (1.0%, pH 6.6) as energy sources, 2) nutrient broth (Difco, Detroit, MI) (pH 7.0), 3) yeast extract, 0.1% (w/v) at pH 3.2 and 6.8, and 4) yeast extract, 0.1%, and glucose, 0.1%, pH 3.2 and 6.8. Tubes were incubated at 28°C and examined weekly for growth visually and by phase contrast microscopy. Positive control tubes were inoculated with *T. ferrooxidans* and *T. thiooxidans* (9K based media only).

Pyrite leaching rates. Pyrite (2% pulp density) was leached in 250 ml shake flasks containing 50 or 100 ml of 9K salts, in some cases diluted to one-tenth strength in 0.01N H_2SO_4. *T. ferrooxidans* (American Type Culture Collection no. 13661) was purified by successive single colony isolations on ISP agar medium and was shown to be free of acidophilic heterotrophs following procedures recommended by Harrison (1984). The organism was grown in 9K medium (ferrous sulfate as energy source) to mid-to-late logarithmic phase of growth and cells were harvested by centrifugation (6,000 x g, 15 min). Cells were washed twice and resuspended in a few ml of 0.01N sulfuric acid in a sterile test tube. The washed cells were separated from the most of the iron precipitates collected during centrifugation by allowing the iron precipitates to settle for 2-4 hours and removing the cells in the supernatant by pipette. Additional 0.01N sulfuric acid was added to the iron precipitate and shaken vigorously, and the above process was repeated. Aliquots of the combined suspension were added to flasks containing pyrite and the flasks were incubated at 28°C in a gyratory shaker at 200 r.p.m. Sample aliquots were removed with time and centrifuged at 12,000 x g for 3 min in a microcentrifuge. Total iron was measured by o-phenanthroline colorimetric method (ASTM, 1987) or by atomic absorption spectrophotometry. Rates of pyrite leaching were calculated from the linear portion of the leaching curve. Sulfate was determined by a turbidimetric procedure (ASTM, 1987).

RESULTS AND DISCUSSION

Analysis of Pyrite

Mineralogical. Mineralogic analysis of a sample of the as received, -10 mesh Waldo pyrite was conducted by the Department of Mining, Environmental and Geological Engineering, New Mexico Tech, Socorro, NM. The material was 95-96% pyrite, 3.5-4% specular hematite (occurring as blades within pyrite and as intergrowths with scant magnetite) and < 0.5% chalcopyrite (occurring as inclusions within pyrite or scant sphalerite). Traces of magnetite, sphalerite and galena were present (G. Griswold, pers. commun., 1988).

X-ray diffraction. X-ray powder patterns showed the material contained mostly pyrite. Smaller peaks were identified as silicon oxide (quartz). There was no evidence of marcasite in the samples .

Fourier transform infrared spectroscopy. This technique was employed to determine the presence of non-pyritic components in the research material such as carbonates and oxidized iron products (jarosite). The infrared spectra of several bottles of pyrite were analyzed and gave similar results. A peak at 1450 cm^{-1} was assigned to residual carbonate in the material. Broad peaks at around 1100 to 1000 cm^{-1} may represent S=O and O-H absorbances in jarosite (Lazaroff *et al.*, 1982) and may also include Si-O. Peaks at 797 and 870 cm^{-1} may represent O-H absorbance in FeOOH. Following bioleaching, the peak assigned to carbonate disappeared as did the peaks at 870 and 1000 cm^{-1} (Figure 1).

Chemical analysis. The pyrite material contained 47.0 %Fe (2.0% r.s.d.) and 42.4 %S (6.6% r.s.d.) by weight from chemical analysis (following peroxide fusion) of pyrite in three bottles. The sulfur value should be viewed as an underestimation since the fusion method was shown to recover only 91% of sulfur from zinc sulfide (Stallings *et al.*, 1988). SEM-EDX analysis showed Fe and S as the major components, with smaller peaks appearing for Si and Al. The Al signal may result from Al in SEM stubs. Traces of Zn, Ca, Mg, and Cu were detected (Figure 2). X-ray fluorescence determination of the chemical composition of the pyrite is in progress.

Microbiological Studies

The pyrite material (six bottles tested) contained no microorganisms capable of growing in 9K salts with ferrous iron (pH 2.5), elemental sulfur (pH 3.5) or thiosulfate (pH 7) as energy sources. Neither was growth detected in nutrient broth (pH 7) or in yeast extract or yeast extract-glucose medium (pH 3 or 7) after 4 weeks of incubation. Positive control tubes inoculated with *T. ferrooxidans* and *T. thiooxidans* (9K media only) showed growth after one week of incubation.

The leaching rate of the pyrite research material was determined in 4 experiments conducted over a 6-month period (Table 1). As with previous studies involving a South Carolina pyrite (Olson, 1988), the pyrite leaching rate was reasonably reproducible in experiments conducted under identical conditions one month apart (6.09 and 6.95 mg Fe/l/hr). Leaching rates were approximately the same in full strength or one tenth strength 9K salts. However, reduction of medium volume from 100 to 50 ml per flask (pulp density 2% in both cases) resulted in faster leaching rates, suggesting gas transfer limitations with larger volumes. However, leaching experiments with a South Carolina pyrite showed that baffled flasks inhibited slightly the bioleaching rates as compared to conventional conical flasks (data not shown).

Leaching rates were calculated from the linear portion of the curve which showed production of soluble iron with time (Figure 3). Generally, this condition occurred during the first two weeks of the experiments. After this time, the rates began to slow, coinciding with a drop in the pH (initially about 2.0) to about 1.7. Experiments were terminated after one month, at which time the pH had dropped to about 1.4-1.5 and total iron in solution was about 3000 mg/l. Control (uninoculated) flasks contained less than 500 mg Fe/l with pH of 2.3-2.4. Some pyrite leaching rates by *T. ferrooxidans* strains reported in the literature ranged from 2.5 to 25 mg Fe/l/hr (Wakao *et al.*, 1984; Kandemir, 1984; Vuorinen and Tuovinen, 1987; Mahapatra *et al.*, 1985). In some cases I calculated these rates from graphs of leaching kinetics in the papers.

Vuorinen *et al.* (1983) studied the occurrence of different iron species in solution during pyrite bioleaching. Total iron in culture solutions was determined by atomic absorption spectrophotometry with and without hot sulfuric acid digestion. Iron concentrations determined on non-digested samples (designated as dissolved and complexed iron by Vuorinen *et al.*, 1983) were 95-99% of those determined after sulfuric acid digestion (termed total iron) in experiments with initial pH values of 2.0. On several occasions during the present study, atomic absorption spectrophotometric determinations of iron in solution (dissolved and complexed iron by Vuorinen *et al.*, 1983) corresponded closely to values determined by the colorimetric procedure. In addition, mass balances of 90% were routinely achieved when the weight loss of pyrite was compared to the sum of iron and sulfate in solution. This indicates that the solution measurements by colorimetric methods are adequate to detect iron and sulfate.

CONCLUSIONS

Iron bioleaching rates from a pyrite research material were determined reproducibly using a defined procedure. The availability of standards will facilitate data intercomparison of bioleaching rates and assist in the identification of superior strains and ores for bioleaching processes.

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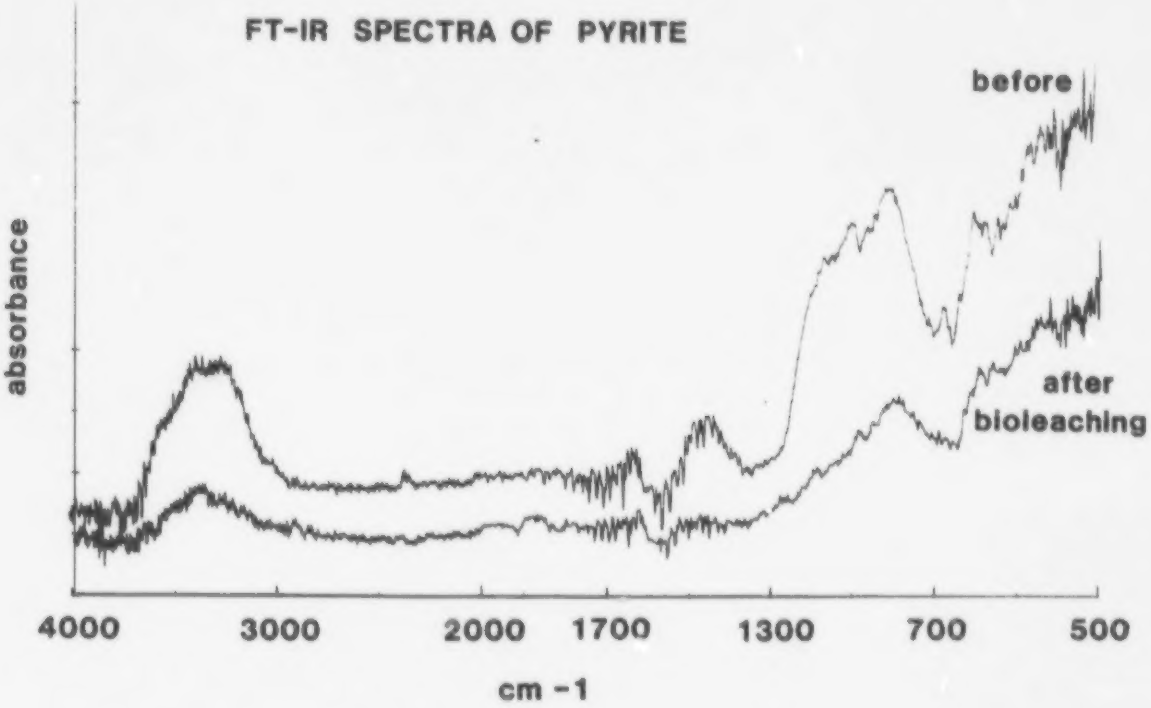

Fig. 1 Infrared (absorbance) spectra from pyrite research material before and after bioleaching.

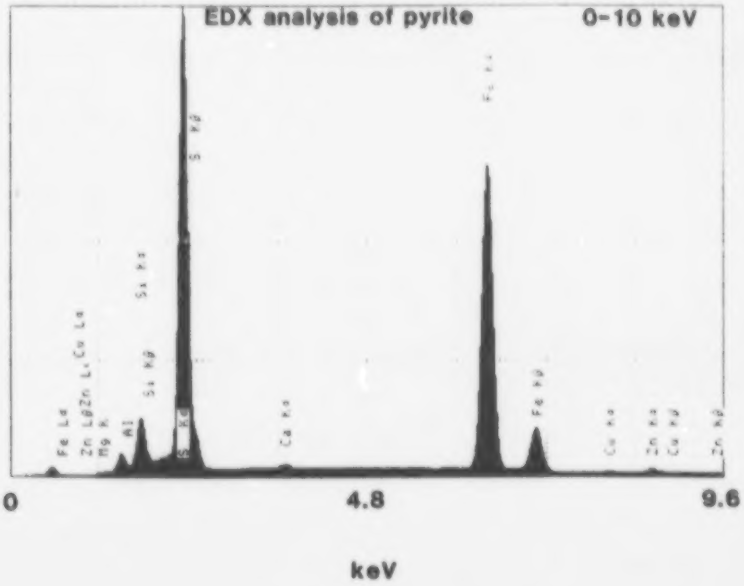

Fig. 2 Energy dispersive x-ray microanalysis of pyrite research material.

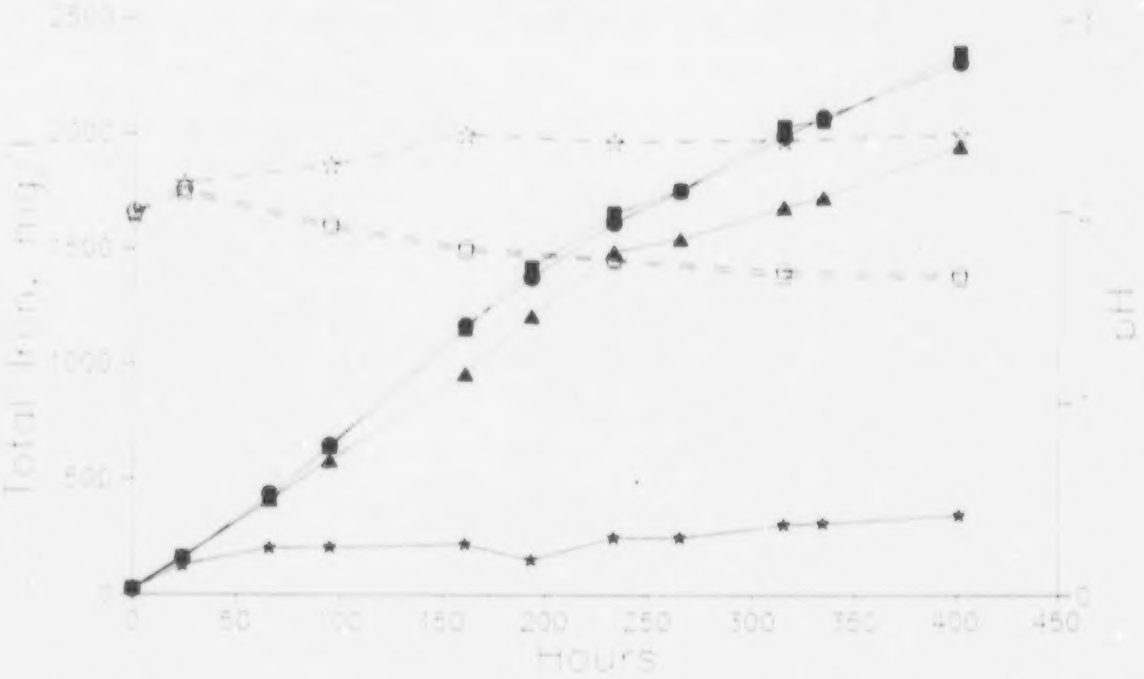

Fig. 3 Bioleaching of pyrite research material by *T. ferrooxidans* . Solid symbols connected by solid lines represent soluble iron from triplicate flasks and a single uninoculated control flask. Open symbols connected by dashed lines represent pH.

Table 1

Bioleaching of New Mexico Pyrite (*T. ferrooxidans*, ATCC 13661)

FeS2,	size	medium	T0 cells/ml	T0 pH	Rate (mg Fe/l/hr)	% r.s.d
1g,	74-149 μm	50 ml 9K salts	2.5×10^8	2.3	3.08	0.7
2g,	58-91 μm	100 ml 9K	3.4×10^8	1.9	6.04	6.4
2g,	58-91 μm	100 ml 0.1 9K	3.4×10^8	1.8	6.09	13.9
2g,	58-91 μm	100 ml 0.1 9K	2.3×10^8	2.0	6.95	8.4
1g,	58-91 μm	50 ml 0.1 9K	1.4×10^8	2.0	13.2	7.0

Rates are means of triplicate determinations in 250 ml conical flasks at 28°C with agitation at 200 r.p.m. on a gyratory shaker.

STABLE ISOTOPE COMPOSITION OF SULPHATE PRODUCED DURING BACTERIAL OXIDATION OF VARIOUS METAL SULPHIDES

W.D. Gould, R.G.L. McCready,* S. Rajan,* and H.R. Krouse***

ABSTRACT

The isotopic composition of sulphate produced during the oxidation of four sulphide minerals by two environmental isolates of *Thiobacillus ferrooxidans* was determined. The substrates were pyrite (FeS_2), sphalerite (ZnS), chalcopyrite ($CuFeS_2$) and pentlandite $(FeNi)_9S_8$. The bacterial oxidation of each of these minerals was carried out in water of varying initial ^{18}O compositions, and samples for sulphate analysis were collected at appropriate time intervals. Insignificant sulphur isotope selectivity was observed during during bacterial oxidation, but the ^{18}O composition of the sulphate was dependent upon that of the water. Since the sulphur oxidizing system of *Thiobacillus thiooxidans* has previously been shown to be a true oxygenase, the incorporation of oxygen from the water into sulphate is probably due to rapid ^{18}O exchange between water and reactive sulphur intermediates.

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* CANMET, Department of Energy Mines and Resources, Ottawa, Ont., Canada, K1A 0G1.

** Department of Physics, University of Calgary, Calgary, Alberta, Canada, T2N 1N4

COMPOSITION ISOTOPIQUE STABLE DU SULFATE RÉSULTANT DE L'OXYDATION BACTÉRIENNE DE DIFFÉRENTS MÉTAUX SULFUREUX

W.D. Gould, R.G.L. McCready*, S. Rajan* et H.R. Krouse***

RÉSUMÉ

La composition isotopique du sulfate résultant de l'oxydation de quatre minéraux sulfureux par deux souches de *Thiobacillus ferrooxidans* isolées de l'environnement, a été déterminée. Les substrats étaient la pyrite (FeS_2), la sphalérite (ZnS), la chalcopyrite ($CuFeS_2$) et la pentlandite ($(FeNi)_9S_8$). L'oxydation bactérienne de chacun de ces minéraux a été réalisée dans de l'eau de compositions initiales en ^{18}O variées, des échantillons pour l'analyse du sulfate ont été recueillis à intervalles appropriés. On a observé pendant l'oxydation que la sélectivité de l'isotope du soufre était négligeable, mais que la composition en ^{18}O du sulfate était dépendante de celle de l'eau. Comme il a en été démontré précédemment que le système d'oxydation de *Thiobacillus thiooxidans* était une vraie oxygénase, l'incorporation d'oxygène de l'eau au sulfate est probablement attribuable à l'échange rapide de ^{18}O entre l'eau et les intermédiaires soufrés réactifs.

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* CANMET, Énergie, Mines et Ressources Canada, Ottawa (Ontario) Canada

** Département de Physique, Université de Calgary, Calgary (Alberta) Canada

INTRODUCTION

Oxygen isotope data may aid in the elucidation of the enzymatic mechanisms of bacterial sulphur oxidation (Suzuki, 1965b), and also provide information about redox reactions in the sulphur cycle (Taylor and Wheeler, 1984; van Everdingen and Krouse, 1985). Under ambient conditions the oxygen exchange rates between sulphate ions and water are very slow (Lloyd, 1968). Thus, the oxygen isotope composition of sulphate produced by the oxidation of sulphur should reflect the original source of the oxygen. However, the exchange of oxygen atoms between sulphate ions and water is much more rapid at acid pH values (Hoering and Kennedy, 1957; Mizutani and Rafter, 1969). Suzuki (1965b) found little or no incorporation of $^{18}O_2$ into thiosulphate during the oxidation of sulphur by *Thiobacillus thiooxidans*. Suzuki (1965a,b) concluded from biochemical data that the enzyme oxidizing sulphur to thiosulphate is a true oxygenase, and the lack of $^{18}O_2$ incorporation into thiosulphate was due to rapid ^{18}O exchange between the water and a reactive oxidized sulphur intermediate. In subsequent studies, Suzuki and Silver (1966) established that sulphite was the initial product of bacterial sulphur oxidation, and that thiosulphate was formed by a chemical side reaction between sulphide and sulphite ions. Taylor *et al.* (1984) calculated that 87.5% of the oxygen incorporated into sulphate during the bacterial oxidation of sulphides was derived from molecular oxygen. In view of the results obtained by Suzuki (1965b) and the potential for isotopic exchange between water and sulphur intermediates, it is not possible to predict the proportion of oxygen in sulphate that is derived from each source. Van Everdingen and Krouse (1985) commented on a method for determining the ^{18}O isotope effects during the bacterial oxidation of sulphides to sulphate but it was based on the stoichiometry of assumed chemical reactions and related only to pyrite oxidation.

The objective of this research was to elucidate the origin of the oxygen incorporated into sulphate during the oxidation of several metal sulphides by two strains of *Thiobacillus ferrooxidans*.

MATERIALS AND METHODS

Substrates:

The following substrates were used in this study: (1) pyrite; Fe 44.36%, S 50.40%, (2) sphalerite; Zn 67.17%, S 32.76%, (3) pentlandite; Fe 37.68%, Ni 14.76%, S 31.22%, (4) chalcopyrite; Fe 30.70%, Cu 34.50%, S 34.37%, (5) HBM & S ore; Fe 26.85%, Zn 0.87%, Ni 0.03%, Cu 0.29%, S 28.07%. The minerals were obtained from Ward's Natural Science Ltd., Mississauga, Ont. They were ground to minus 100 mesh (ASTM), pretreated with 0.1 N HCl and then washed with deionized water.

REAGENTS AND MICROORGANISMS:

The $H_2{}^{18}0$ enriched water was obtained from Isotec Inc., Dayton, Ohio. One of the *Thiobacillus ferrooxidans* isolates (EL-5) was isolated from a mine water sample (McCready *et al.* , 1986). The other isolate of *T. ferrooxidans* (SM-4) was obtained from Dr. I. Suzuki of the University of Manitoba (Lizama and Suzuki, 1988). The inocula were maintained on the HBM & S ore (6 g ore + 100 mL autotrophic medium) in 250 mL Erlenmeyer flasks at 30°C on a rotary shaker (150 rpm). The autotrophic medium used during this study was a sulphate free medium of the following composition: K_2HPO_4 0.1 mM; $MgCl_2$ 0.5 mM; NH_4Cl 1.0 mM, adjusted to pH 2.5.

EXPERIMENTAL DESIGN:

The experiments were carried out in 2.8 liter Fernbach flasks and contained 10 g of sulphide mineral, 900 mL of media, and 100 mL of inoculum. Varying amounts of $H_2{}^{18}O$ enriched water (10% enriched) were also added to some of the samples. The flasks were incubated at 30°C on a rotary shaker (150 rpm).

The media were made up using either Calgary water (δ^{18}O, - 17.5‰ or Ottawa water (δ^{18}O, -11.1‰) enriched by the addition of varying amounts of $H_2{}^{18}O$ (10% enriched). Nine initial levels of ^{18}O enriched media had δ^{18}O values varying from + 17.1‰ to + 61.4‰. Six experiments were run using the following substrates: (1) 10 g pyrite, (2) 10 g sphalerite, (3) 10 g pentlandite (4) 10 g chalcopyrite, (5) 5 g chalcopyrite + 5 g pentlandite, (6) 10 g pyrite. Isolate EL-5 was used for experiments 1 to 5 and isolate SM4 was used for experiment #6.

ANALYTICAL METHODS:

After various time intervals, samples of the supernatant (80 mL unless otherwise specified) were withdrawn and filtered twice through Whatman #42 filter paper. Ten mL of the filtrate was used for metal analysis and 25 to 50 mL of the filtrate for sulphate determination. The metal ion concentrations were analyzed by atomic absorption spectrophotometry using an Instrumentation Laboratories IL 551 instrument. Four mL of concentrated HCl was added to the aliquot taken for sulphate analysis, and the solution was heated to a boil. Then 10 mL of 10% w/v $BaCl_2$ was added to the acidified filtrate and the precipitated $BaS0_4$ was allowed to settle overnight. The barium sulphate was filtered and collected on a 0.45μ Millipore filter, washed with 100 mL of 1 N HCl, 500 mL of deionized water and dried for 24 h at 60°C. The dried $BaS0_4$ was weighed and transferred to small vials for subsequent δ^{18}0 and δ^{34}S determinations.

MASS SPECTROMETRY:

$BaSO_4$ was converted to SO_2 for sulphur isotope analysis using the high temperature decomposition procedure of Ueda and Krouse (1987).

For oxygen isotope analyses, $BaSO_4$ was reduced by graphite in platinum boats through which currents of the order of 20A were passed. Both CO_2 and CO were produced in this high temperature reaction. The CO was converted to CO_2 using a high voltage discharge unit and a liquid N_2 bath. The apparatus is a variation of that described by Sakai and Krouse (1971).

Sulphur and oxygen isotope analyses were obtained with mass spectrometers that have been constructed around Micromass 602 and Micromass 903 basic components.

RESULTS

The fraction of ^{18}O incorporated into sulphate from water was approximately equal to the slope of the line obtained by plotting δ^{18}O (SO_4) versus δ^{18}G (H_2O) (Figure 1). Between 55 and 75% of the oxygen incorporated into sulphate during the bacterial oxidation of pyrite by *Thiobacillus ferrooxidans* isolate EL-5 originated from water (Table 1). The amount of oxygen incorporated from water was found to increase with time. Soluble iron concentrations increased for 13 days then decreased slightly for the next two sampling times (Figure 2). The amount of sulphate recovered continued to increase for the duration of the experiment. A similar trend in the δ^{18}O data was observed during the bacterial oxidation of pyrite by isolate SM-4 (Table 1, Figure 3).

The bacterial oxidation of sphalerite occurred rapidly (Figure 4) and somewhat less oxygen was incorporated from water (50-64%) (Table 1) than during the oxidation of pyrite. Approximately 68-75% of the oxygen in sulphate was derived from water during the bacterial oxidation of pentlandite (Table 1). The oxidation of this mineral was also rapid (Figure 5). A much lower incorporation of ^{18}O from the water into the sulphate (23-48%) was observed during the bacterial oxidation of chalcopyrite (Table 1). The oxidation rate of this mineral was very slow (Figure 6). The ^{18}O incorporation pattern and the oxidation rate of the mixture of pentlandite and chalcopyrite were similar to those of pentlandite alone (Table 1, Figure 7).

DISCUSSION

The decrease in the soluble iron concentration observed during the oxidation of pyrite by isolate EL-5 may be due to the precipitation of various iron oxyhydroxide species (Figure 2). The proportion of the oxygen in sulphate derived from water (23 to 80%) during the bacterial oxidation of sulphide minerals in our experiments agrees with the results obtained by other workers. Taylor *et al.* (1984b) found 23 to 65% of the oxygen in sulphate produced during the bacterial oxidation of pyrite to be incorporated from water. Van Everdingen *et al.* (1985) calculated that 37 to 74% of the oxygen in environmental sulphate samples was derived from water. The chemical oxidation of pyritic sulphur has been postulated to occur by direct attack of the ferric ion on the metal sulphide and addition of a hydroxyl radical to the sulphur (Moses *et al.* 1987). By this mechanism, all of the oxygen in the sulphate would be derived from water.

The lower incorporation of oxygen from water into sulphate during the bacterial oxidation of chalcopyrite must be viewed cautiously. Due to the very slow oxidation rate of this mineral, 40% of the sulphate at the conclusion of the experiment was introduced from the inoculum (Figure 6). Corrections have been made for the contribution of the inoculum to the final isotopic composition and in the case of chalcopyrite, the assigned error is larger than for other minerals. It is noted, however, that the slope steadily increased during the course of the experiment. An unknown is the concentration of intermediates in the transferred inoculum. It is also not known how much of the oxygen in the SO_4 produced from carry-over of intermediate was determined by water in the inoculum or the reactor.

This study shows that a significant portion of the oxygen in the sulphate generated during the bacterial oxidation of sulphide minerals is derived from water, but the mechanism by which this occurs is yet to be determined. The incorporation of oxygen from water into the sulphate may occur by enzymatic addition, analogous to the chemical oxidation by ferric ions, or by isotopic exchange between an enzyme-bound, oxidized sulphur intermediate and water. The oxidation of reduced sulphur compounds by the thiobacilli can occur by several different pathways (Hooper and DiSpirito, 1985; Kelly, 1982). The enzymes sulfite reductase (Tuovinen *et al.*, 1976) and APS reductase (Vestal and Lundgren, 1971) have been shown to be absent in *T. ferrooxidans*. Thus the oxidation of sulphides by *T. ferrooxidans* probably occurs via the sulphide oxidase and sulphite oxidase enzyme systems. In the absence of detailed reaction mechanisms for the enzymatic oxidation of reduced sulphur compounds it would be difficult to predict at which reaction step(s) isotopic exchange would occur. Andersson *et al.* (1982) using ^{15}N NMR analysis and oxygen-18 labelled intermediates observed very rapid oxygen exchange between nitrite and water during the oxidation of ammonia to nitrite by *Nitrosomonas*. They observed no appreciable oxygen exchange between nitrite ions and water in the absence of bacteria. They estimated that at least one of the oxygens incorporated into ammonia by *Nitrosomonas* originated from dioxygen (Andersson *et al.*, 1982; Andersson and Hooper, 1983). However, subsequent rapid exchange between water and an oxidized nitrogen intermediate resulted in the oxygen of the nitrite reflecting the isotopic composition of the water. We have also observed the incorporation of $^{18}O_2$ into both sulphate and water during the oxidation of sulphide minerals by *T. ferrooxidans* (unpublished results). Thus, these results are consistent with the incorporation of molecular oxygen into the sulphur intermediate followed by oxygen exchange between a sulphur intermediate and water. Future studies should be directed towards determining the potential for oxygen exchange between oxidized sulphur intermediates and water during specific enzymatic reactions that are involved in the bacterial oxidation of reduced sulphur compounds.

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TABLE 1

Dependence of oxygen isotope composition of sulphate on that of water during the bacterial oxidation of sulphide minerals by *Thiobacillus ferrooxidans*. [$\delta^{18}O(SO_4^{2-}) = m\delta^{18}O(H_2O) + b$]

SUBSTRATE	ISOLATE	TIME, DAYS	SLOPE, m	INTERCEPT, b
Pyrite	EL-5	6	0.55 ± 0.02	2.3 ± 1.6
		13	0.63 ± 0.02	4.5 ± 1.5
		20	0.66 ± 0.03	4.5 ± 2.1
		27	0.73 ± 0.02	3.1 ± 1.5
		34	0.75 ± 0.02	3.6 ± 1.1
		Average	0.66 ± 0.02	3.6 ± 2.6
Pyrite	SM-4	7	0.80 ± 0.03	3.7 ± 2.6
		14	0.75 ± 0.03	4.3 ± 2.2
		21	0.63 ± 0.05	4.7 ± 3.7
		28	0.67 ± 0.03	3.3 ± 2.6
		35	0.71 ± 0.04	2.5 ± 3.4
		Average	0.71 ± 0.02	3.7 ± 3.2
Sphalerite	EL-5	6	0.50 ± 0.02	5.9 ± 1.6
		13	0.64 ± 0.03	5.4 ± 2.2
		20	0.59 ± 0.03	8.2 ± 2.1
		27	0.61 ± 0.04	7.5 ± 2.9
		34	0.53 ± 0.05	9.8 ± 3.8
		Average	0.57 ± 0.02	7.7 ± 3.2
Pentlandite	EL-5	6	0.68 ± 0.01	7.1 ± 1.0
		13	0.70 ± 0.01	7.2 ± 0.9
		20	0.69 ± 0.03	7.0 ± 1.8
		27	0.75 ± 0.02	6.5 ± 1.6
		Average	0.70 ± 0.01	7.0 ± 1.4
Chalcopyrite	EL-5	6	0.23 ± 0.05	3.1 ± 4.1
		13	0.32 ± 0.07	4.5 ± 5.2
		27	0.38 ± 0.05	5.0 ± 3.9
		41	0.48 ± 0.05	4.6 ± 3.6
		55	0.46 ± 0.06	5.3 ± 4.6
		Average	0.37 ± 0.03	4.5 ± 5.0
Pentlandite + Chalcopyrite	EL-5	7	0.80 ± 0.02	7.2 ± 1.7
		14	0.66 ± 0.04	10.0 ± 2.9
		21	0.68 ± 0.04	9.8 ± 3.0
		28	0.68 ± 0.01	8.8 ± 1.0
		35	0.68 ± 0.04	9.9 ± 3.4
		42	0.76 ± 0.02	7.1 ± 1.4
		Average	0.71 ± 0.01	8.8 ± 2.6

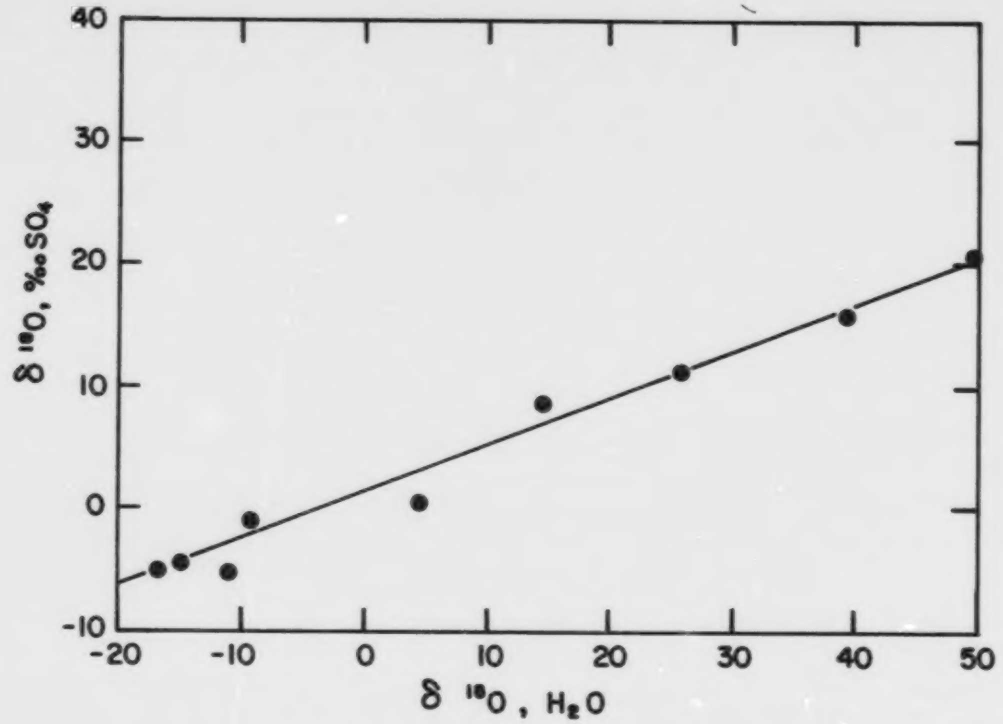

Fig. 1 $\delta^{18}0$ values of sulphate produced during the oxidation of pyrite by isolate EL-5 in water of varying $\delta^{18}0$ composition.

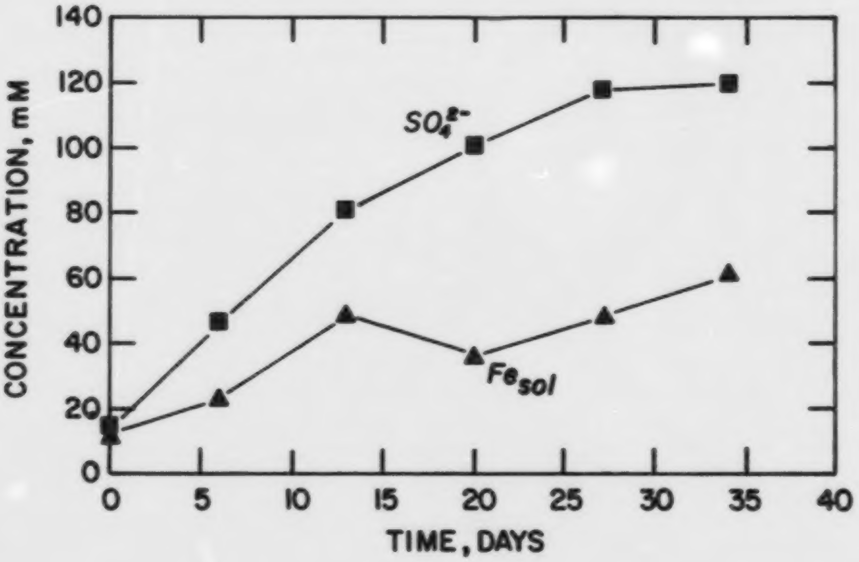

Fig. 2 Soluble metal and sulphate concentrations at various time intervals during the bacterial oxidation of pyrite by *T. ferrooxidans* (isolate EL-5). Fe_{sol}-total soluble iron.

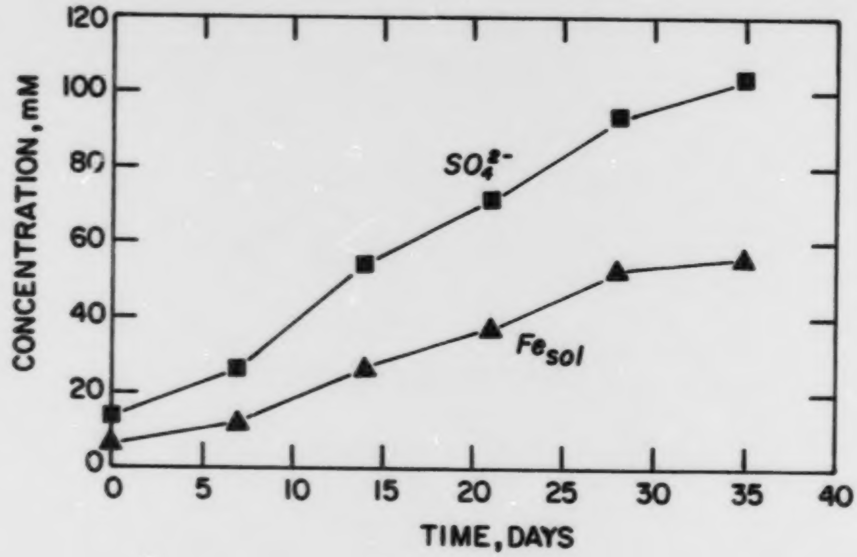

Fig. 3 Soluble metal and sulphate concentrations at various time intervals during the bacterial oxidation of pyrite by T. ferrooxidans (isolate SM-4). Fe_{sol}-total soluble iron.

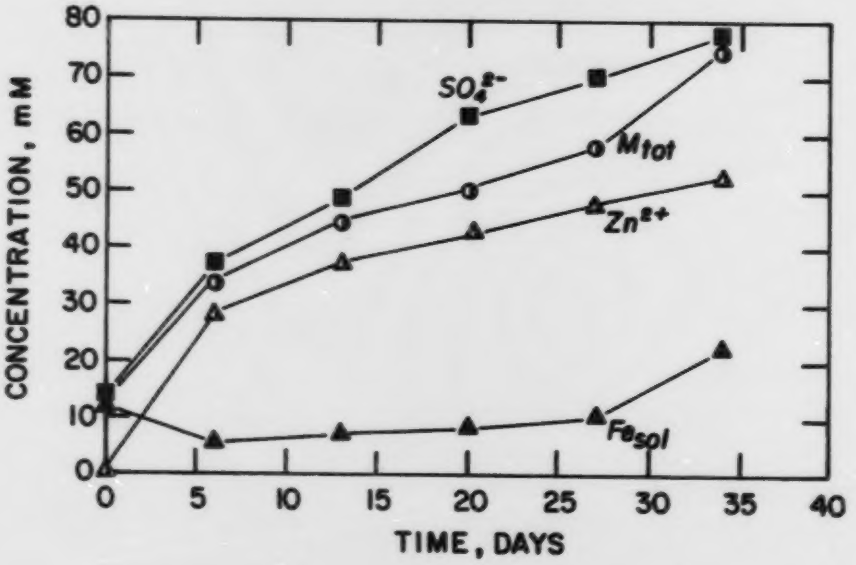

Fig. 4 Soluble metal and sulphate concentrations at various time intervals during the bacterial oxidation of sphalerite by *T. ferrooxidans* (isolate EL-5). M_{tot}-total metals. Fe_{sol}-total soluble iron.

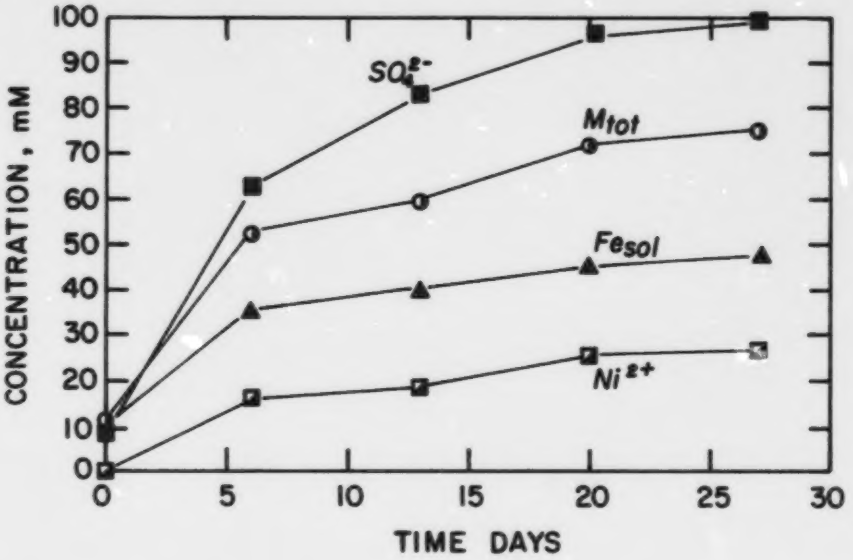

Fig. 5 Soluble metal and sulphate concentrations at various time intervals during the bacterial oxidation of pentlandite by *T. ferrooxidans* (isolate EL-5). M_{tot}-total metals. Fe_{sol}-total soluble iron.

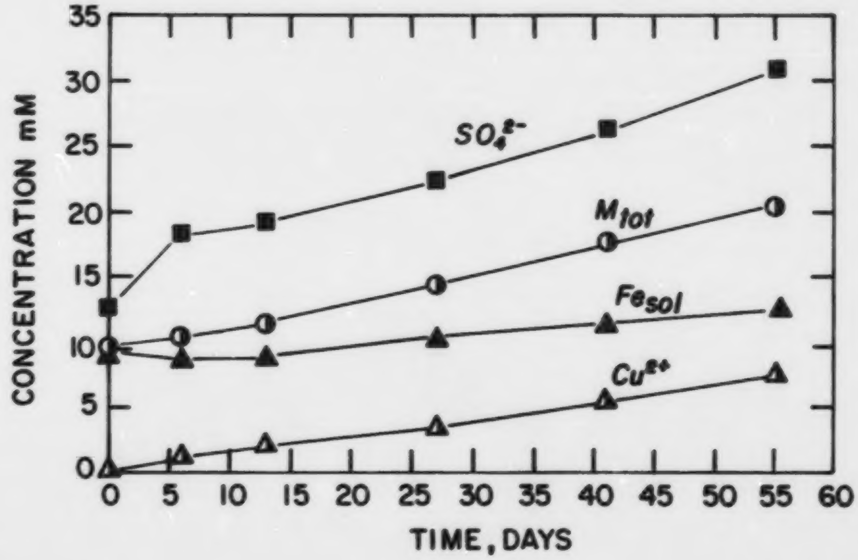

Fig. 6 Soluble metal and sulphate concentrations at various time intervals during the bacterial oxidation of chalcopyrite by *T. ferrooxidans* (isolate EL-5). M_{tot}-total metals. Fe_{sol}-total soluble iron.

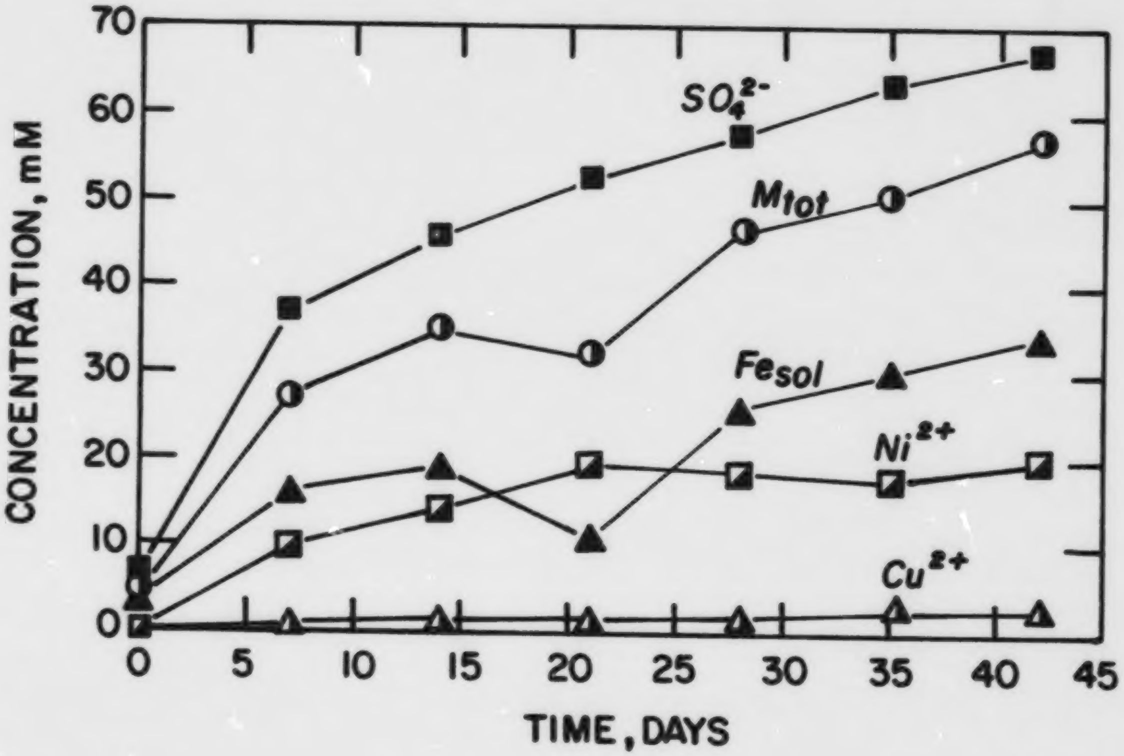

Fig. 7 Soluble metal and sulphate concentrations at various time intervals during the bacterial oxidation of a mixture of pentlandite and chalcopyrite by *T. ferrooxidans* (isolate EL-5). M_{tot}-total metals. Fe_{sol}-total soluble iron.

MICROBIOLOGICAL PROCESSING OF BAUXITE

by

G.I. KARAVAIKO[1], Z.A. AVAKYAN[1], L.V. OGURTSOVA[1] AND O.F. SAFONOVA[2]

ABSTRACT

Three applications of microorganisms to dressing of bauxite are discussed: removal of silicon, removal of iron, and destruction of aluminogoethite. The mechanism and kinetics of these processes depend on the nature of the microorganisms and the metabolites they produce as well as on the properties of bauxites to be processed. It was shown that removal of silicon from kaolinite-hematite-gibbsite bauxite by *Bacillus mucilaginosus* was based on a process of selective separation of the kaolinite-enriched fine fraction rather than on kaolinite destruction and solubilization. The silicon module of bauxite increased in the process from 2.49 to 5.02-5.38. Removal of iron from kaolinite-goethite-gibbsite and siderite-kaolinite-gibbsite bauxites depended on the organic acids, mainly citric acid, which were produced by *Aspergillus niger* and lowered the iron content by 29% and 71%, respectively. Pretreatment of aluminogoethite-containing bauxites with *A. niger* and *Aureobasidium pullulans* resulted in alumino-geothite destruction and an increase in the release of aluminium into alkaline-aluminate solution by 3.6%.

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[1] Institute of Microbiology, USSR Academy of Sciences, Moscow, USSR.

[2] Institute MEKHANOBR, Leningrad, USSR.

TRAITEMENT MICROBIOLOGIQUE DE LA BAUXITE

G.I. Karavaiko[1], *Z.A. Avakyan*[1], *L.V. Ogurtsova*[1] *et O.F. Safonova*[2]

RÉSUMÉ

Trois applications des micro-organismes pour le traitement de la bauxite, notamment l'extraction de la silice, l'extraction du fer et la destruction de l'aluminogoéthite sont discutées. Le mode d'action et la cinétique de ces procédés dépend de la nature des micro-organismes et des métabolites qu'ils produisent ainsi que des propriétés des bauxites à traiter. Il est démontré que l'extraction par *Bacillus mucilaginosus* de la silice de la bauxite formée de kaolinite-hématite-gibbsite est basée sur le procédé de séparation sélective de la fraction riche en kaolinite plutôt que sur la destruction ou la mise en solution de la kaolinite. La concentration de la silice dans la bauxite passe de 2,49 à 5,02-5,38 pendant le traitement. L'extraction du fer des bauxites constituées de kaolinite-goéthite-gibbsite et de sidérite-kaolinite-gibbsite dépend principalement des acides organiques, notamment de l'acide citrique, qui sont produits par *Aspergillus niger* et qui réduisent la teneur en fer respectivement de 29 pour cent et de 71 pour cent. Le prétraitement des bauxites renfermant de l'aluminogoéthite par *A. niger* et par *Aureobasidium* pullulans provoque une destruction de l'aluminogoéthite ainsi qu'une augmentation de libération d'aluminium de 3,6 pour cent dans une solution d'aluminate alcaline.

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[1] Institut de Microbiologie, Académie des Sciences de l'U.R.S.S., Moscou, Russie

[2] Institut MEKHANOBR, Leningrade, Russie

INTRODUCTION

Bauxites consist mainly of aluminium minerals such as gibbsite, boehmite, diaspore; iron minerals such as goethite and hematite; aluminosilicates such as kaolinite of residual and secondary origin; and some other minerals. The quality of bauxites is characterized by the silicon module (alumina/silica ratio by weight). The widely used Bayer process utilizes only bauxites with a silicon module of 7 or higher, which are rare in nature.

The prospects for using microorganisms in the dessing of bauxite have been insufficiently studied. Some authors used strains of *B. mucilaginosus* (Andreev *et al.*, 1976) and *Bacillus circulans* (Groudeva a. Groudev, 1984), which synthesize exopolysaccharides, to remove silicon from kaolinite-containing bauxites. It has been reported that these bacteria dissolve silicate minerals and solubilize silicon. It has also been suggested that a specific group of "silicate" bacteria exists which utilize energy from the destruction of silicate minerals (Yakhontova *et al.*, 1986).

The anlysis of the data available in literature throws doubt on such concepts (Karavaiko *et al.*, 1984; Avakyan *et al.*, 1986; Avakyan, 1988). These bacteria — *B. mucilaginosus* and strains of *B. circulans* — belong to heterotrophs and are incapable of developing in the absence of organic matter (Avakyan *et al.*, 1986; Groudev a. Groudeva, 1988). Their metabolites determine the character of the effect on minerals while the kinetics of the processes triggered by them depend on the process conditions and type of minerals.

This investigation was aimed at studying the prospects for using different microorganisms in the dressing and processing of bauxite.

METHODS

Different types of bauxites from deposits in the USSR, Hungary and Guinea were used in the experiments.

Microorganisms and Experiment Conditions Some Microorganisms were isolated from different types of rocks. Others were received from the All-Union Collection of Microorganisms (VKM). To study their role in the destruction of bauxite minerals the following media were used (g.L^{-1}); sucrose, 20; $(NH_4)_2SO_4$, 0.1; $MgSO_4$, 0.2; K_2HPO_4, 0.5; tap water, 1.L; pH 7.8 for *B. mucilaginosus* VKM B-148OD; and sucrose, 20; $(NH_4)_2SO_4$, 1; $MgSO_4$, 0.2; K_2HPO_4, 1; NaCl, 0.1; tap water, 1 L; pH 7.0 for mycelial micromycetes and yeasts.

The experiments were performed in 250- or 500- mL Erlenmeyer flasks at 10% pulp density. An ore sample ground to 70% size- class of -0.074 mm was added to the nutrient medium and sterilized at 0.5 atm for 30 min.

An inoculum in the amount of 10% was added as a suspension of spores of mycelial micromycetes or as a suspension of vegetative cells of bacteria and yeasts.

In all experiments, controls with sterile microorganism-free medium were run.

The flasks were placed on a rotary shaker (280 rpm) at 30°C. The duration of experiments varied from 7 to 28 days.

Chemical and Physical Analyses Al_2O_3, SiO_2 and Fe_2O_3 in solution as well as in solid samples after calcination were assayed using atom absorption spectrophotometry on an AAS-1 spectrophotometer (GDR).

X-ray analysis were performed in an DRON-UM-1 unit (Co radiation).

The grain size composition was determined by size class using screen analysis for sizes over 0.044 mm. A FRITSCH laser analyzer was used for sizes under 0.063 mm; and an ultrasound analyzer ("Rotup") for sizes over 0.063 mm.

The viscosity of the medium with exopolysaccharides of *B. mucilaginosus* was determined with a viscosimeter.

RESULTS

Bauxite Dressing Low-grade bauxite dressing is aimed at selectively decreasing the silicon content and increasing the silicon module. The more extented experiments were performed as follows: *B. mucilaginosus* was cultivated in the medium with hematite-kaolinite-gibbsite bauxite. Every 7 days the culture liquid was separated from the solid phase by decantation and fresh nutrient medium was added. The development of *B. mucilaginosus* was accompanied with production of exopolysaccharides which increased the medium viscosity (ν_{rel} = 2.18-3.5). The medium decreased to 5.4-5.5.

SiO_2 and Al_2O_3 content of the decanted culture liquid was determined, and suspended fine fractions of bauxites which did not settle during 1 hour were assayed. They were separated from the solution by centrifuging at 25,000 x g during 20 min.

The fine fraction of bauxite separated from the decanted solution after 48 hours had a higher level of silicon and a lower level of aluminium (Table 1). In contrast, the larger fraction of bauxite that had settled during the sedimentation had a lower level of silicon and a higher level of silicon module. The level of soluble forms of Al_2O_3 and SiO_2 in the solution after the separation of suspended fines did not exceed 5-10 and 20-80 $mg \cdot L^{-1}$, respectively, and which did not affect the silicon module of bauxite.

In a longer experiment (28 days) in which the medium was replaced 4 times, 5.4% of bauxite by weight was decanted as a suspensing of fine particles while the silicon module of the solid residue rose from 2.40-2.50 to 5.0-5.40.

Thus, the removal mechanism of silicon from hematite-kaolinite-gibbsite bauxites by *B. mucilaginosus* and its metabolites was based on a process of separation of fine silicon-rich fractions of bauxites by exopolysaccharides rather than on the process of kaolinite destruction and silicon solubilization.

To evaluate the suitability of this method for removal of silicon from other types of bauxites, experiments were performed using 14 samples of hematite-chamosite-diaspore, kaolinite-chamosite-boehmite, kaolinite-hematite-boehmite, gibbsite-kaolinite-boehmite and chamosite-hematite-boehmite bauxites from several deposits in the USSR. The experiments and analyses of solutions were performed as described above. It was found that after the bacterial treatment of these bauxites, the fine fraction was also separated with the decanted solution. The chemical composition of the fine fraction, however, was identical to that of the bauxite in the solid residue. Table 2 presents the data for hematite-goethite-boehmite bauxite which clearly show that the silicon module did not change if the fine fraction is removed by decantation, nor in the solid residue after the decantation.

It is obvious that the minerals composition of bauxites played a decisive role in removal of silicon from bauxites. The data of chemical analyses of different size classes of bauxites show (Fig. 1) that in hematite-kaolinite-gibbsite bauxite (sample 1209) the highest silicon module (M_{Si} = 6.76) was associated with the largest size class (>0.25 mm), and the module sharply decreased in finer size classes. In other types of bauxites, this pattern was reversed: for example, in chamosite-boehmite bauxite (sample 59) the highest silicon module (M_{Si} = 6.8) was associated with the finest fractions (size class <0.005 mm), and the silicon module sharply decreased in larger size classes. A similar pattern of the silicon module behaviour in different size classes was observable after bacterial attack.

It is clear that in bauxites ground to 70% size- class of -0.074 mm used in these experiments, the fine silicon-rich particles of kaolinite were selectively separated from hematite-kaolinite-gibbsite bauxity by exopolysaccharides produced by *B. mucilaginosus*. This technique of removal of silicon was not suitable for processing other types of bauxites studied.

Removal of Iron Another possible approach to upgrading bauxites is the selective removal of iron using microorganisms. The effect on bauxites of 63 strains of autotrophic and heterotrophic microorganisms belonging to 15 genera was studied. The highest rate of iron leaching was displayed by *A. niger* 4 which was used in further experiments.

In 35-day experiments with periodic replacement of the nutrient medium (every 7 days), the release of SiO_2, Al_2O_3 and Fe_2O_3 from different bauxites exposed to *A. niger* 4 was determined. The results presented in Table 3 show that all principal elements were solubilized, which precluded an increase in the silicon module in the residue. However, in some cases iron was selectively removed. For example, iron was preferentially leached from kaolinite-goethite-gibbsite and siderite-chamosite-gibbsite bauxites while in the experiment with kaolinite-hematite-boehmite bauxite mostly silicon and iron were solubilized which slightly increased the silicon module.

It was noted that hematite-kaolinite-gibbsite bauxite which was dressed by *B. mucilaginosus* was resistant to processing by metabolites from mycelial fungi. This type of bauxite as well as gibbsite-kaolinite-boehmite and hematite-goethite-boehmite bauxites were the most refractory to attack by fungal metabolites. This suggests a low level of destruction of the constituent minerals, including kaolinite.

However, mycelial fungi seem promising for dressing some types of bauxites (siderite-kaolinite-gibbsite and kaolinite-goethite-gibbsite) by removing iron.

Aluminogoethite Destruction Some laterite bauxites display a high silicon module but contain, besides iron minerals – goethite and hematite – also iron oxides with isomorphic aluminium admixtures. Aluminium was not recovered from aluminogoethite when bauxites were processed using the Bayer method, and was lost with the sludge, decreasing the recovery rate of Al_2O_3 by 3-10%. The effect of microorganisms on aluminogoethite and aluminohematite had not been studied previously.

The experiments used hematite-goethite-gibbsite bauxite (Debel deposit, Guinea) assaying 17% aluminogoethite. Isomorphic substitution of iron with aluminium in these bauxites averaged 27 molar %. Bauxite samples were exposed to growing cultures of microorganisms belonging to the genera *Aspergillus*, *Penicillium*, *Aureobasidium*, *Candida*, *Pseudomonas*, *Bacillus*, *Arthrobacter*. All strains under study displayed different activity in the destruction of aluminogoethite, the most active strains being *A. spergillus* 4 and *A. pollulans*.

The typical signs observable during the development of *A. niger* were a decrease in pH to 1.9 as a result of citric acid production (90% of total organic acids) and sorption of fine bauxite particles on mycelial globules.

The growth of *A. upeobdsidium* was also accompanied by a decrease in medium pH to 2.5 and production of the exopolysaccharide pollulan which was excreted into the medium. This increased the viscosity of the medium, ensuring that fine bauxite fractions were suspended in the culture liquid together with *A. pollulans* cells and did not settle during 1 hour of sedimentation.

Taking into account the pattern of distribution of different bauxite fractions in the pulp, the fine bauxite fractions adsorbed by *A. niger* mycelium and those suspended in the culture liquid of *A. pollulans* which was settled by centrifuging at 25,000 x g for 20 min as well as bauxite fractions not associated with microorganisms were analyzed separately.

Figure 2 compares the sections of diffractograms corresponding to the input bauxite and bauxite exposed to *A. niger* and *A. pollulans*. The decrease in the peak intensity of aluminogoethite from d = 0.413 nm can be clearly seen, especially in the fine fractions absorbed by *A. niger* mycelium or suspended in the culture liquid of *A. pollulans*. Concurrent, with the decrease in the peak value, the "d" value of the latter increased slightly to 0.414-0.416 nm indicating a decrease in isomorphic incorporation of aluminium into the aluminogoethite lattice to 10-22 molar % Al_2O_3 in the experiment with *A. niger*.

The data from the chemical analysis of different bauxite fractions treated with microorganisms show that the level of aluminium oxide both in the fine fraction sorbed by microorganisms and, to a lesser extent, in the residue not sorbed by microorganisms, decreased (Table 4). This indicates solubilization of aluminium. To prevent losses of Al_2O_3, the resultant solution should be utilized in the subsequent processes to produce alumina from bauxites. As a result of bauxite treatment with microorganisms, the adsorbed or suspended fine bauxite fraction was enriched in SiO_2. The silicon module of fine fractions was decreased while the silicon module of bauxites not sorbed by microorganisms was increased.

Because of the lower levels of aluminium and silicon in bauxites treated with microorganisms, the relative level of iron was increased.

To evalute the degree of aluminogoethite destruction and the technical significance of this process, bauxite treated with microorganisms and containing both fractions was leached by means of the Bayer process. The extraction of Al_2O_3 into solution when pretreated bauxites were used increased by 3.6% while the aluminium oxide content of the red sludge went down to 10.6% as compared to the sludge from a control which assayed the 14.27% Al_2O_3.

The experiments revealed the possibility of using microorganisms for the treatment of aluminogoethite-containing bauxites to increase the extraction of aluminium in alumina prodocution by means of the Bayer process.

CONCLUSION

The experiments performed in this investigation have shown that there is no single approach to the application of microorganisms in processing of bauxites. This is attributed to the diversity of mineral and chemical composition of bauxites.

The application of microbial exopolysaccharides (for example, those produced by *B. mucilaginosus*) to treat bauxites with kaolinite-rich fine fractions seems to be attractive for separating those fractions from the total mass of bauxites. In this case, bacterial exopolysaccharides help to separate bauxite fractions by removal of the fine silicon-rich fraction as an undesirable admixture.

Metabolites such as citric acid synthesized by mycelial fungi are promising for solubilizing iron minerals contained in bauxites. Solubilized aluminium can be partially recycled into the alumina production by means of the Bayer technique.

Finally, a possible third approach is related to aluminogoethite destruction. This mineral is present, for example, in high-grade bauxites from Guinea. Pretreatment of these bauxites with *A. niger* and *A. pollulans* increased the release of aluminium into alkaline-aluminate solution.

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Table 1

Chemical analysis of different fractions of hematite-kaolinite-gibbsite bauxite after treatment with *B. mucilaginosus* VKM B-1480D (experiment period, 48 hours).

Bauxite fraction	Element content (%)			M_{si}
	Al_20_3	$Si0_2$	Fe_e0_3	
Fine fraction of bauxite from solution	34.0	15.90	12.20	2,10
Bauxite after solution decantation	42.20	10.70	26.30	3,90
Bauxite in control	45.70	18.10	19.90	2,5

Table 2

Chemical analysis of different fractions of hematite-goethite-boehmite bauxite after treatment with *B. mucilaginosus* (experiment period, 28 days).

Bauxite fraction	Element content (%)			M_{si}
	Al_20_3	$Si0_2$	Fe_e0_3	
Fine fraction of bauxite from solution	41.78	13.50	25.32	3.08
Bauxite after solution decantation	41.60	13.58	24.18	3.06
Bauxite in control	42.87	13.07	27.78	3.2

Note: Chemical analysis of the input bauxite (%): Al_20_3, 41.78; $Si0_2$, 12.54; Fe_20_3, 27.78.

Table 3

Leaching of $Si0_2$, Al_20_3 from different types of bauxites by *A. niger* 4

Bauxite type	Chemical composition of input bauxites (%)			Total extraction of elements in 35 days ($mg \cdot l^{-1}$). Medium was replaced every 7 days			M_{si} (numerator denominator)
	$Si0_2$	Al_20_3	Fe_20_3	$Si0_2$	Al_20_3	Fe_20_3	
Hematite-chamosite-diaspore	17.83	45.08	18.1	3390	1070	1152	2.61/2.53
Hematite-kaolinite-gibbsite	17.85	44.39	18.78	360	34 5	388	2.63/2.49
Boehmite-goethite-kaolinite	25.22	60.04	7.79	640	29 0	49	2.20/2.38
Gibbsite-kaolinite-boehmite	19.33	57.03	14.72	500	32 5	86	2.87/2.95
Gibbsite-kaolinite-goethite	10.94	37.40	29.94	495	6 62	4657	3.26/3.08
Kaolinite-gibbsite-goethite	16.26	52.50	4.76	498	186 2	2122	
Siderite-kaolinite-gibbsite	13.54	40.03	11.87	475	81 0	8587	2.95/2.78
Kaolinite-hematite-boehmite	10.06	56.02	20.85	1850	5 45	1780	6.56/5.66

Table 4

SiO_2, Al_2O_3 and Fe_2O_3 content in aluminogoethite-containing bauxite treated with microorganisms

Sample	Chemical composition (%) (after calcination)				M_{si}
	Al_2O_3	ΔAl_2O_3	SiO_2	Fe_2O_3	
Bauxite treated with *A. pullulans*	47.89	-2.93	2.62	31.57	22.10
Fine fraction from decanted culture liquid of *A. pullulans*					
1st decantation	47.37	-13.52	4.0	36.44	11.84
2nd decantation	53.69	-7.20	3.50	30.22	15.34
3rd decantation	56.58	-4.24	3.75	29.90	15.09
Bauxite treated with *A. niger*	56.62	-4.47	2.32	32.05	24.41
Fine fraction adsorbed by *A. niger* mycelium	55.35	-5.47	3.73	34.65	14.84
Bauxite treated with sterile medium without microorganisms	60.82	–	3.14	24.21	19.37

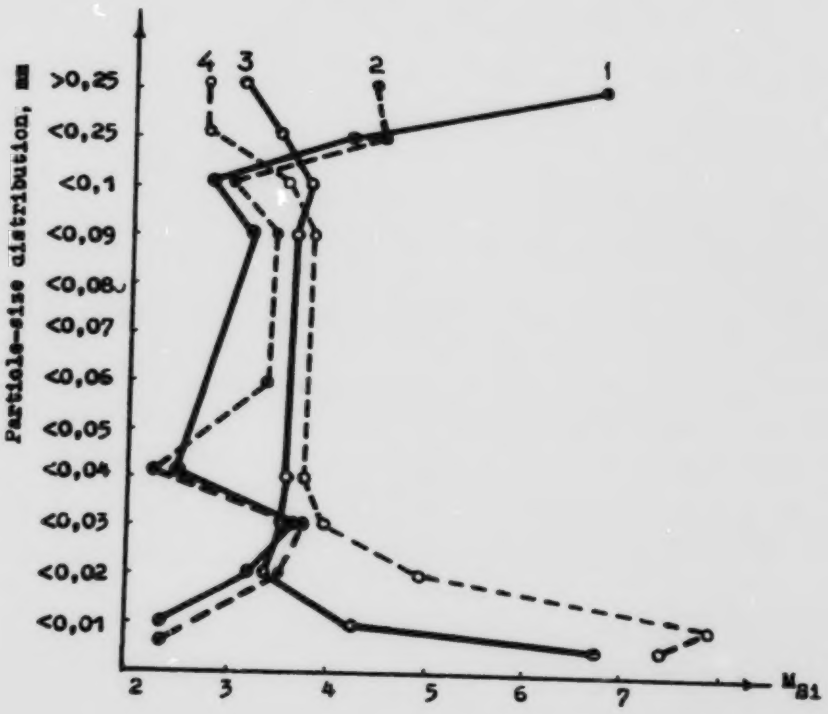

Fig. 1. Changes in the silicon module (M_{si}) of bauxites depending on the size class

1 – goethite-kaolinite-gibbsite bauxite before bacterial treatment

2 – goethite-kaolinite-gibbsite bauxite before bacterial treatment

3 – kaolinite-chamosite-boehmite bauxite before bacterial treatment

4 – kaolinite-chamosite-boehmite bauxite before bacterial treatment

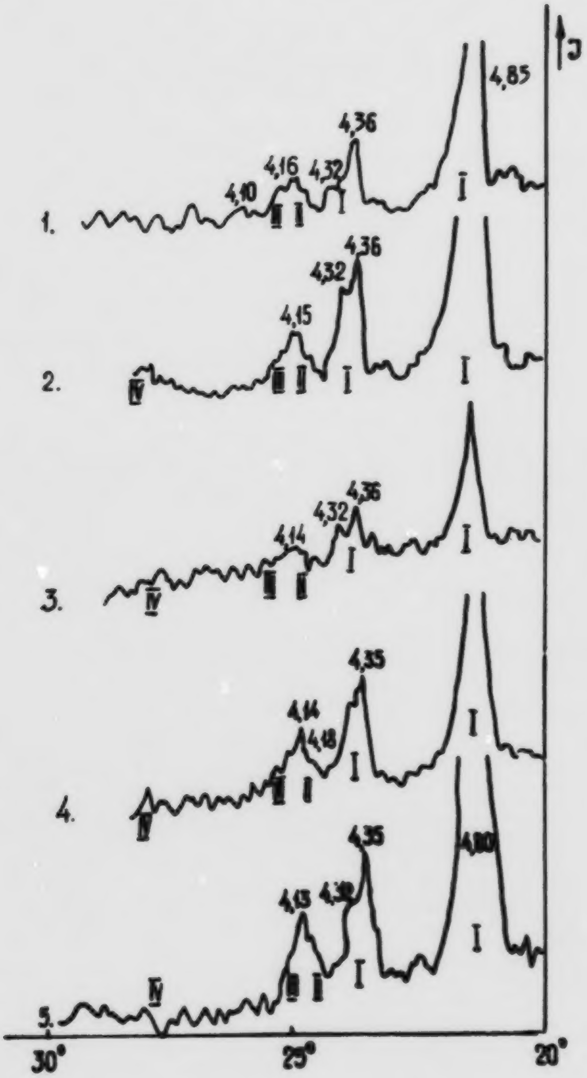

Fig. 2. Diffractograms of the input and microbially treated aluminogoethite-containing bauxites

1 — bauxite adsorbed by *A. niger*

2 — bauxite not adsorbed by *A. niger*

3 — bauxite suspended in the culture liquid of *A. pullulans*

4 — bauxite not adsorbed by *A. pullulans*

5 — input bauxite

I — gibbsite, II — goethite, III — aluminogoethite, IV — hematite

GENETICS STUDIES

THE GENETIC MANIPULATION OF BACTERIA FOR BIOLEACHING

Douglas E. Rawlings

Department of Microbiology, University of Cape Town,
Rondebosch 7700, South Africa

ABSTRACT

The rate of pyrite oxidation by natural isolates of *Thiobacillus ferrooxidans* may be too slow to permit the economically competitive leaching of certain ores or concentrates. Mutation and selection, and recombinant DNA technology (genetic engineering) are two approaches that may be used to improve the performance of *T. ferrooxidans* strains. Each approach has distinct advantages and disadvantages, and it would be of great value to be able to use both techniques. To date only the mutation and selection approach has been used successfully. Although much of the information necessary for the application of recombinant DNA techniques to *T. ferrooxidans* has recently become available, not all of the problems associated with using this approach have been resolved. A summary of the requirements necessary for the genetic engineering of *T. ferrooxidans* is given, and a discussion of the elements in place and those still missing is presented.

LA MANIPULATION GÉNÉTIQUE DE BACTÉRIES POUR LA BIOLIXIVIATION

Douglas A. Rawlings

Département de Microbiologie Université de Cape Town,
Rondebosch 7700, Afrique du Sud

RÉSUMÉ

Le taux d'oxydation de la pyrite par des isolats naturels de *Thiobacillus ferrooxidans* peut être trop faible pour rendre la lixiviation de certains minerais ou concentrés compétitive économiquement. La mutation et sélection, et la technologie de l'ADN recombinant (génie génétique) sont deux approches qui peuvent être utilisées pour améliorer la performance des souches de *T. ferrooxidans*. Chaque approche comporte des avantages et des désavantages, il serait bien de pouvoir combiner ensemble ces deux techniques. Jusqu'à présent l'approche de mutation et sélection a été utilisée avec succès. Même si une quantité importante de l'information nécessaire à l'application des techniques de l'ADN recombinant chez *T. ferrooxidans* est maintenant disponible, plusieurs problèmes doivent encore être résolus. Un résumé des conditions à remplir pour appliquer le génie génétique chez *T. ferrooxidans* ainsi qu'une discussion portant sur ce qui est connu et sur ce qui ne l'est pas sont présentés.

INTRODUCTION

Bioleaching is one of the economically proven options for the recovery of metals from copper, uranium and more recently gold-bearing pyrite/arsenopyrite ores. Biological treatment methods may have considerable advantages over the alternative physical/chemical processes, particularly with respect to energy consumption and environmental impact. A major difficulty with biological processes is that bioleaching rates are often too slow for the process to be economically competitive. Populations of leaching bacteria that have recently been isolated from nature should however not be expected to oxidize ores at maximum rates. Natural populations of bacteria would have been genetically selected for their ability to survive under a wide range of often adverse conditions, rather than for their ability to rapidly oxidize ores under the ideal conditions of an industrial bioleach process. The challenge to the biologist is to improve the rate of growth and ore oxidation by the microbes responsible for leaching. If this can be achieved, the bioleaching option may become the most cost effective and environmentally compatible method of treatment.

GENETIC IMPROVEMENT OF NATURAL ISOLATES OF BIOLEACHING ORGANISMS

Although it has been found that several species of bacteria are capable of leaching metals from ores and that in general mixed cultures of bacteria do so better than pure cultures, *Thiobacillus ferrooxidans* is still considered to play the major role in most industrial leaching operations. There are two approaches to the genetic improvement of *T. ferrooxidans* and other populations of leaching criteria. These are: a) mutation and selection and b) genetic engineering. Each approach has distinct advantages and disadvantages and ideally it should be possible to use either technique.

Mutation and selection

Mutation and selection can be as easily applied to mixed cultures of bacteria as to pure cultures. The technique is dependant on the small number of spontaneous errors in the DNA sequence that are made when the chromosome of a bacterium is replicated. The frequency of errors may be increased through the use of mutagens which interfere with the fidelity of the DNA replication mechanism and raise the mutation rate above the natural level. Most mutations are neutral or harmful, but some that are advantageous to the cell will occasionally occur. If a selective pressure is placed on a mutating microbial population, those bacteria that have acquired a mutation that has enabled them to outcompete the rest will eventually dominate the population. Mutation and selection is a simple, low technology procedure. For example, the rate of growth and oxidation by a population of leaching bacteria can be improved simply by cultivating a population of the bacteria in a continuous flow apparatus and then slowly increasing the flow rate. Those bacteria that are capable of the most rapid growth will be subject to less washout than the others and will be preferentially selected.

The main advantages of mutation and selection are firstly, that it is a low technology procedure which can be applied in any basic laboratory. Secondly, unlike genetic engineering, the technique is not necessarily dependent on an in-depth understanding of the biochemistry of the bacterium (although this may help). Disadvantages are that it may take a long time to improve the bacteria, to an economically significant extent. Furthermore, since mutation and selection is essentially an empirical technique, very little information on the bacterium being manipulated is gained. This approach has, nevertheless been used to improve the leach rates of *T. ferrooxidans* dominated isolates approximately 10 fold over that of the original isolates. This has had a substantial effect on the economics of the bioleach option. Some of these improved populations are being used very successfully in the full-scale treatment of ores and concentrates (van Aswegen *et al.*, 1988).

Genetic Engineering

An important difference between mutation and selection and genetic engineering is that during mutation and selection no new genetic material is acquired by the bacteria. The procedure merely selects for modifications in proteins or regulatory systems already present in the bacteria. If a bacterium does not possess a certain ability because it lacks the genetic material to do so, mutation and selection will not enable the organism to acquire that property. In contrast, genetic engineering results in the placement of new genetic material in the organism being manipulated.

Another advantage of genetic engineering is that multiple copies of the organism's own DNA can be placed into a cell. This could result in increased expression of the genes contained on the multiple-copy DNA. Once a genetic system is in place, genetic engineering should be a more rapid procedure than mutation and selection. However, unlike mutation and selection, a substantial amount of information about the biochemistry of the bacterium to be modified is required before genetic engineering is possible.

Genetic engineering is not a general technique that can be applied to a mixed culture of bacteria. Each bacterium that plays a significant role in the bioleaching process would have to be engineered separately. Because of its importance in the bioleaching process, *T. ferrooxidans* is the obvious candidate for the first round of genetic engineering.

REQUIREMENTS FOR A GENETIC SYSTEM

Three basic requirements for the use of recombinant DNA techniques on a genetically unstudied bacterium such as *T. ferrooxidans* are illustrated in Figure 1.

These are:

i) a plasmid cloning vector which is known to be able to replicate and be stably maintained in *T. ferrooxidans*.

ii) the identification of a gene that can be spliced into the plasmid and which codes for a property (genetic marker) that can be expressed in *T. ferrooxidans* . This enables those bacteria that have acquired the gene to be identified among the many others that have not.

iii) a method for introducing genetically manipulated DNA into *T. ferrooxidans*.

The difficulty in developing a genetic system for a relatively unstudied organism like *T. ferrooxidans* is that none of these basic requirements have been met. Although bacterial cloning vectors and selectable genetic markers are available for other bacteria, it is not known which of the cloning vectors will be replicated in *T. ferrooxidans* or which genetic markers will be expressed. Most bacterial plasmids are not replicated in unrelated bacteria, and genes from some bacteria are poorly or not at all expressed in others. In addition there is no universally applicable technique for introducing DNA into bacteria. Since *T. ferrooxidans* has a unique physiology and occupies an ecological niche populated by few other bacteria, it is difficult to predict to which bacteria *T. ferrooxidans* is genetically related. With so many unknowns, if an attempt to introduce foreign DNA into *T. ferrooxidans* was unsuccessful, it would not be known whether the plasmid had failed to be replicated, the gene used as a marker was not expressed or whether the method for introducing the DNA into the cells was unsuitable.

To make progress in the development of a genetic system, it is important to be able to investigate each of these basic requirements independently of each other. One approach to achieving this would be to isolate plasmids and genes from *T. ferrooxidans*. These would be expected to be replicated and expressed when placed back into the bacterium. It would then be possible to work on the introduction of DNA into *T. ferrooxidans* with reasonable certainty that a lack of success would not be due to problems associated with plasmid replication or gene expression.

Plasmids from T. ferrooxidans

T. ferrooxidans plasmids are not only sources of DNA that can replicate in the bacterium but also potential sources of genes that may serve as genetic markers in *T. ferrooxidans* . Plasmids are widespread among natural isolates of *T. ferrooxidans* (Martin *et al.*, 1981). Several workers have isolated and in some cases cloned these plasmids into well-characterized plasmids from other bacteria (Holmes *et al.*, 1984). All *T. ferrooxidans* plasmids studied so far are cryptic. That is, the function of these plasmids and whether they encode any selectable genetic markers is unknown.

One *T. ferrooxidans* plasmid has been identified that has the unusual property of being able to replicate in a wide variety of Gram-negative bacteria. The mechanism of replication of this plasmid is being studied to determine which regions are involved in replication, plasmid stability and copy number control (Dorrington and Rawlings, 1989). This type of plasmid has great potential for use in the development of cloning vectors for *T. ferrooxidans* and other Gram-negative bacteria. There are however other broad-host-range plasmids which have been shown to replicate in all the Gram-negative bacteria in which they have been tested, and it is likely that these could be equally well suited for use in *T. ferrooxidans*.

Genetic markers for T. ferrooxidans

Several genes from *T. ferrooxidans* have been isolated which could serve as potential genetic markers for the development of a *T. ferrooxidans* genetic system. However, to be of value it would be necessary to mutate and inactivate the copy of the gene on the *T. ferrooxidans* chromosome.

Techniques for the production and isolation of suitably defined auxotrophic mutants of *T. ferrooxidans* have still to be developed.

Shiratori *et al.* (1989) have recently reported the cloning of mercury ion resistance genes from *T. ferrooxidans* and their expression in *E. coli*. This is the first selectable genetic marker to be isolated from *T. ferrooxidans* and should prove useful in the development of a *T. ferrooxidans* transformation system since it is almost certain to be expressed in the bacterium. Attempts have been made to use genetic markers from other Gram-negative bacteria without success. These include the resistance genes for chloramphenicol, ampicillin and tetracycline (on sulfur media).

Introduction of DNA into T. ferrooxidans

There are four methods for introducing recombinant DNA into bacteria. These are the well-studied techniques of transduction, transformation and conjugation, and the less well-studied technique of electroporation.

Transduction involves the use of bacterial viruses (phages) to deliver DNA to a sensitive cell. As no phages that infect *T. ferrooxidans* have been reported, it is not possible to use this technique.

Transformation is a process whereby some bacteria become competent to take up naked DNA during a particular phase of their growth cycle either naturally or when artificially induced to do so. A very limited range of bacteria have a natural ability to be transformed and the conditions for inducing competence artificially vary substantially among bacteria.

Protocols for transformation are usually developed empirically and critical conditions for transformation are most often discovered serendipitously. Attempts to transform *T. ferrooxidans* using a variety of techniques have not been successful. The same applies to attempts to transform *T. ferrooxidans* spheroplasts (bacteria from which most of the cell walls have been removed). This lack of success does not mean that *T. ferrooxidans* is not transformable, but that further work needs to be carried out to establish the correct conditions for the production of competent cells. The successful transformation of some bacterial genera was only achieved once a transformable strain was discovered.

Conjugation or bacterial mating is a broadly applicable means by which plasmids may be transferred between bacteria that belong to the same or even different genera. *T. ferrooxidans* plasmids have been identified which are capable of being conjugated with the aid of other "helper" plasmids. Several workers have attempted to conjugate both *T. ferrooxidans* plasmids and broad-host-range plasmids between laboratory bacteria such as *E. coli* and a variety of thiobacilli including *T. ferrooxidans* (Davidson and Summers, 1983; Kulpa *et al.*, 1983). In some experiments a two-step mating procedure was carried out in which an attempt was made to use non-iron-oxidizing thiobacilli such as *T. novellus* and *T. intermedius* as a bridge between *E. coli* and *T. ferrooxidans* (Rawlings *et al.*, 1986).

Although broad-host-range plasmids including the *T. ferrooxidans* plasmid have been successfully conjugated into non-iron-oxidizing thiobacilli and between non-iron-oxidizing thiobacilli, none has been conjugated into *T. ferrooxidans*. The observation that conjugable broad-host-range plasmids do occur in *T. ferrooxidans* (Rawlings and Woods, 1985) is nevertheless encouraging. It is an indication that the bacterium may be able to conjugate with other as yet unidentified bacteria under the right conditions. The heterotrophic bacterium *Acidiphilium cryptum* which grows in close association with *T. ferrooxidans* may be one such bacterium.

Electroporation is a routine method of delivering DNA into plant and animal cells that has recently been applied to bacteria. The technique involves the use of an electrical discharge to produce pores in the bacterial cell membrane which open briefly to allow DNA into the cell before resealing spontaneously. Although many bacteria may be killed in the process, a small proportion of those that survive acquire the plasmid. No studies on the electroporation of *T. ferrooxidans* have been reported, but preliminary work has indicated that *T. ferrooxidans* is more resistant to the lethal effects of electroporation than most other bacteria (J. Rohrer, pers. commun., 1989). This is an indication that the cell membrane of *T. ferrooxidans* is less disrupted by electroporation than most other bacteria and that higher field strengths than usual may be required.

Comparison of genes from T. ferrooxidans with those of other bacteria

As discussed earlier, one of the major advantages of genetic engineering is that using this technique, it is possible to place new genetic material into a bacterium such as *T. ferrooxidans*. This genetic material could have been isolated from other species of bacteria or even other organisms. Since not all bacteria are able to read and interpret the start (promoter) and stop (termination) signals of genes from other bacteria, it would be useful to know which bacteria are genetically most closely related to *T. ferrooxidans*. If one wishes to introduce into the bacterium a gene from a genetically distant organism, it may be necessary to express the foreign gene using *T. ferrooxidans* control signals. This raises questions as to: i) which bacteria *T. ferrooxidans* is genetically most closely related to, and ii) how the control signals of *T. ferrooxidans* genes compare with those of other bacteria.

In order to address these questions, several genes from *T. ferrooxidans* have been cloned and their expression in easy-to-handle laboratory strains of *E. coli* has been studied. In some cases the DNA sequences of the *T. ferrooxidans* genes and their flanking regions have been determined. These include the genes for the enzymes glutamine synthetase (glnA), nitrogenase (nifHDK) and the recA gene product (Barros *et al.*, 1985; Pretorius *et al.*, 1986; Ramesar *et al.*, 1989). In addition, the gene for the iron-induced copper protein, rustacyanin, has been cloned (Kulpa *et al.*, 1986), and two families of repeated sequences that are found in a number of *T. ferrooxidans* strains have been cloned and one of the families sequenced (Yates *et al.*, 1988). Rustacyanin is unlikely to occur in non-iron-oxidizing bacteria, and repeated sequences are rather rare among bacteria, so although these genes and sequences provide valuable information on the structure of some important *T. ferrooxidans* genetic elements, they are of limited value for comparative purposes. Genes involved in the replication of a *T. ferrooxidans* plasmid have also been sequenced, but these are also of limited comparative value (Dorrington and Rawlings, unpublished).

A comparison of the *T. ferrooxidans* glnA, nifHDK and recA genes with those of other bacteria has recently been made (Rawlings, 1989) and only a brief summary is presented here. The glnA gene product is expressed in *E. coli* from *T. ferrooxidans* transcription signals and has all the properties, including regulation of activity by adenylation, commonly found in Gram-negative bacteria. However, the commonly associated regulatory genes, ntrB and ntrC, do not lie immediately downstream of the glnA gene as found in most other Gram-negative bacteria. When the amino acid sequence of the glnA gene product is compared to that of the other bacteria for which this information is available, *T. ferrooxidans* is approximately equally related to all the other Gram-negative bacteria.

Expression of the nifHDK genes cannot be directly studied in *E. coli* since this organism does not fix nitrogen. However, by constructing a fusion of the nifHDK genes to an easily assayed β-galactosidase gene, it has been possible to study its expression in *E. coli* (D. Berger, pers. commun., 1988). With respect to expression, regulation and gene structure, the *T. ferrooxidans* nifHDK genes appear to be identical to those of most other bacteria. The amino acid sequences of the nifHDK gene products are clearly more closely related to some species of bacteria than others. Based on the amino acid sequence of the nitrogenase iron protein, *T. ferrooxidans* appears most closely related to members of the genus *Bradyrhizobium* and to a lesser extent to members of the genus *Rhizobium*.

The *T. ferrooxidans* recA gene is not expressed from its own start signal in *E. coli*. Whether this is because the *T. ferrooxidans* signal is located further upstream than the 2.2kb of DNA tested or whether it is simply not recognized in *E. coli* is still uncertain. The recA gene product is however, fully functional when expressed from an *E. coli* plasmid-located start signal. Since the *T. ferrooxidans* recA gene is only the fourth recA gene to be sequenced, it is not possible to compare its amino acid sequence with those of a wide range of other bacteria. The *T. ferrooxidans* recA protein is 61%, 66% and 69% homologous to the *Bacteroides fragilis*, (H. Goodman, pers. commun., 1989) *E. coli* and *Pseudomonas aeruginosa* recA proteins respectively.

DISCUSSION

Mutation and selection and genetic engineering should not be seen as two alternative, or mutually exclusive approaches for improving *T. ferrooxidans* strains. Rather, once a genetic system is available, each technique has distinct advantages and disadvantages and it may be necessary to use both. Mutation and selection is a natural and ongoing process. In a continuous-flow process, mutation and selection (or natural adaptation) will be taking place automatically using the spontaneous mutations that arise from errors in DNA replication. Those individual bacteria that can outgrow the rest will eventually dominate the population and form the platform on which the next round of improvement will be based. The bioleach option to processing an ore should not be discarded on the grounds of an initial slow leach rate. Provided that an ore is amenable to leaching, mutation and selection can improve the leach rate many fold. Furthermore, a bacterial culture that efficiently leaches a given ore may not be as efficient at leaching another ore, even though the two ores may be similar. An extended period of mutation and selection (adaptation) may be necessary before the maximum leach rate of the new ore is obtained.

Genetic engineering of *T. ferrooxidans* will not be possible until a method is developed for the introduction of recombinant DNA into the bacterium. In the absence of *T. ferrooxidans* phages, conjugation and electroporation would seem to be the DNA transfer procedures with the greatest potential. Although suitable plasmid vectors are available, the detection of a successful DNA transfer experiment has been dependant on the expression of foreign genetic markers in *T. ferrooxidans*. The recent isolation of the mercury resistance genes from *T. ferrooxidans* means that it will now be possible to attempt transformation with genes isolated from the bacterium. No experiments based on the expression of an homologous genetic marker have yet been reported. In regard to gene expression, although the *T. ferrooxidans* nitrogen metabolism genes glnA and nifHDK were expressed in *E. coli*, these genes are controlled by a special class of nitrogen-regulated start signals. Whether the more typical variety of start signals (-35 to -10 promoters) are also similar to those of other bacteria is still uncertain. It would be of value to isolate strong *T. ferrooxidans* promoter sequences for use in the expression of foreign genes in the organism. The nitrogen regulated promoters currently available are likely to be switched off in the presence of an adequate nitrogen supply and are therefore not ideal.

The use of recombinant DNA techniques to study the biochemistry and physiology of *T. ferrooxidans* should lead to breakthroughs in our understanding of the molecular biology of the organism. Many of these findings could have practical significance. For example, an investigation into the genes involved in the production and regulation of the proteins used in the oxidation of iron and sulfur is a challenge to molecular biologists that could provide insight into how to improve the growth and leach rate of the bacterium. Similarly, a study of the ability of *T. ferrooxidans* to use ferric iron as an alternate electron acceptor to oxygen and how this ability is regulated could be of use in oxygen-limited vat and *in situ* leaching operations.

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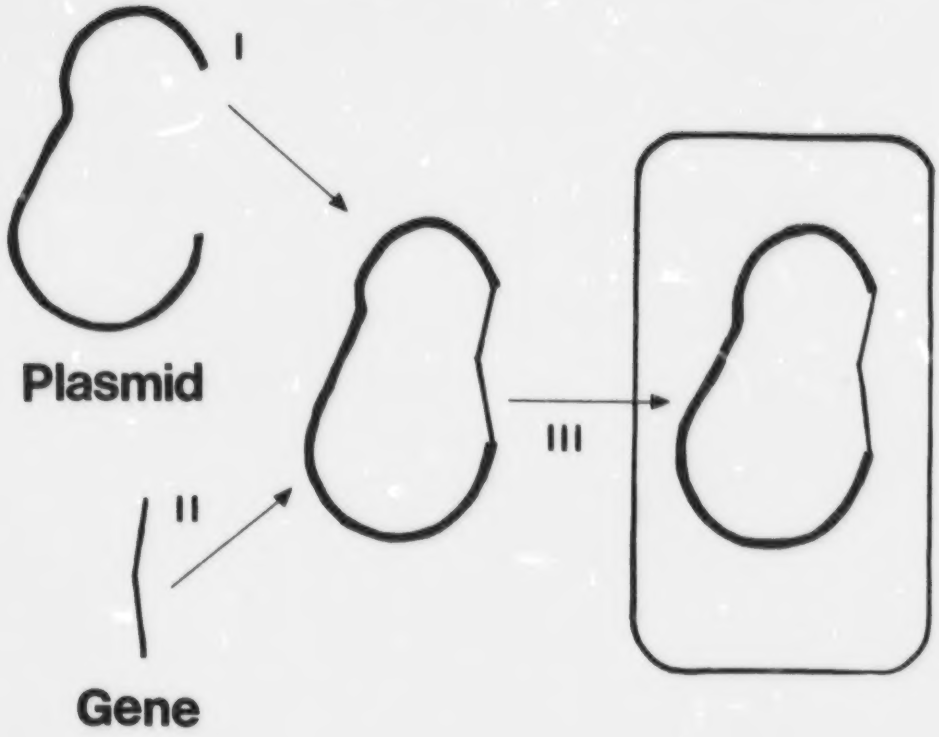
I
Plasmid
II
Gene
III

Fig. 1 The basic requirements for a genetic system for *T. ferrooxidans*.

ADAPTATION OF *THIOBACILLUS FERROOXIDANS* FOR INDUSTRIAL APPLICATIONS

DAVID S. HOLMES and RIAZ UL HAQ

Department of Biology
Rensselaer Polytechnic Institute Troy, NY 12180

SUMMARY

Thiobacillus ferrooxidans ATCC 19859 contains mobile, repetitive DNA sequences of at least two types. One type, termed family 2, has been sequenced and has the physical characteristics of an insertion sequence. The other type, termed family 1, has not yet been sequenced. *T. ferrooxidans* ATCC 19859 can rapidly adapt to a wide range of growth conditions some of which are of commercial significance. This adaptation is associated with specific changes in the chromosomal location of family 1 sequences. Examination of numerous strains of *T. ferrooxidans* from around the world show that both family 1 and family 2 are widely distributed suggesting that such strains might also undergo rapid adaptation to produce novel types of microorganisms for industrial applications.

L'ADAPTATION DE *THIOBACILLUS FERROOXIDANS* À DES FINS INDUSTRIELLES

David S. Holmes et Riaz Ul Haq

Département de Biologie
Institut Polytechnique de Rensselaer Troy, NY 12180 É.-U.

RÉSUMÉ

La souche *Thiobacillus ferrooxidans* ATCC 19859 possède au moins deux types de séquences répétitives mobiles d'ADN. Un type, désigné famille 2, a été séquencé et possède les caractéristiques d'une séquence d'insertion. L'autre type, appelé famille 1, n'a pas encore été séquencé. *T. ferrooxidans* ATCC 19859 peut s'adapter rapidement à une grande variété de conditions de croissance, ce qui peut parfois avoir des conséquences importantes au point de vue commercial. Cette adaptation est associée à des changements spécifiques de localisation des séquences de la famille 1 dans le chromosome. L'analyse d'un grand nombre de souches de *T. ferrooxidans* provenant de sites à travers le monde démontre que les familles 1 et 2 sont très répandues, ce qui suggère que ces souches sont peut-être capables de s'adapter rapidement pour former de nouveaux types de microorganismes potentiellement intéressant pour l'industrie.

INTRODUCTION

Industrial strains of microorganisms can be developed by three procedures; by selection and adaptation of natural occurring strains, by classical genetic approaches such as mutagenesis and conjugation, or finally by genetic engineering techniques. Selection and adaptation of naturally occurring strains has historically been important for the development of microorganisms for toxic waste remediation and dump leaching for metal recovery. However, two main problems exist with this approach. First, it relies on the occurrence of normally rare mutations, and second, the rare mutations that do occur are random and are very unlikely to enhance the properties of the microorganism for a specific application. This naturally leads to very slow progress in strain development.

We recently discovered that *Thiobacillus ferrooxidans* undergoes mutation at a rate that far exceeds normal point mutation rates, due to the presence and mobility within the genome of repetitive DNA elements (3, 12, 19). In one instance there is preliminary evidence that a particular mutation, the loss of iron-oxidizing activity, is associated with the movement of a repetitive DNA sequence into a defined location within the genome (4).

Because of this enhanced mutation rate we wished to evaluate whether industrial strains of *T. ferrooxidans* could be developed by adaptation and selection within reasonable periods of time. In this paper we extend our observations regarding the mobility of repetitive DNA elements. We also demonstrate that changes in the genomic location of such elements are associated with the development of a novel strain of *T. ferrooxidans* of industrial interest. Finally, a survey shows that the repetitive elements are found in many strains of *T. ferrooxidans* from around the world suggesting that the occurrence of repetitive DNA is important for the survival of the species and that such strains might also undergo rapid adaptation to produce novel microorganisms for industrial applications.

MATERIAL AND METHODS

Bacterial Strains

T. ferrooxidans: ATCC 19659, ATCC23270 (type strain), ATCC 14119, ATCC 33020 and ATCC 13661 were obtained from the American Type Culture Collection (ATCC), Rockville, MD 20852. ATCC 13661H and ATCC 13661K are clonal derivatives of ATCC 13661 isolated in our laboratory (H) or the laboratory of Dr. C. Kulpa (K). Strains Duval, Torma, Pyrite, Penn Coal and LL(TM) were obtained from Dr. H. Ehrlich, Rensselaer Polytechnic Institute, Troy, NY 12180. DSM 583 was obtained from the Deutche Sammlung von Mikrooganismen(DSM), Gottingen, FRG. Strains YS14, YS14S, YSG6, SHI LS, SHI H, SHI J, SHI L and SCI A were isolated from acid mine drainage (gift of Dr. R. Unz, Pennsylvania State University, University Park, PA 16802) in our laboratory. Strain TF 203 was obtained from Dr. A. Agate, MACS Institute, Pune, India.

Other bacterial strains: The following were obtained from the ATCC: *T. thiooxidans*, ATCC 19377 (type strain), ATCC 8085 and ATCC 15494; *T. thioparus*, ATCC 80815 (type strain); *T. novellus*, ATCC 8093 (type strain); *Acidiphilium cryptum*, ATCC 33463 (type strain).

A. organovorum, ATCC 43141 (type strain) was isolated in our laboratory. *Leptospirillus ferrooxidans*, Z2 (type strain) and Duval were obtained from H. Ehrlich, and *T. acidophilus*, ATCC 27807 (type strain) and DSM 700 were obtained from the ATCC and DSM respectively.

Media

T. ferrooxidans was grown on solid 9K-ferrous iron medium pH 2.4 (1,2), solidified with agarose as described previously (3). Liquid 9K-ferrous iron medium pH 2.4 was prepared as described above but omitting the agarose. Liquid thiosulfate medium (4) was made as described previously (5).

T. thiooxidans was grown in liquid "Thiobacillus" medium as described in the ATCC catalogue. *Leptospirillum ferrooxidans* was maintained in liquid 9K-ferrous iron medium as described above except the pH was adjusted to 1.6. *A. cryptum* and *A. organovorum* were grown as described previously (6). *T. thioparus* was grown in S-6 medium + 1% $Na_2S_2O_3$ as described in the ATCC catalogue. *T. novellus* and *E. coli* were grown in nutrient broth.

DNA Preparation

High molecular weight genomic DNA from *T. ferrooxidans* and *L. ferrooxidans* was prepared by a modification of the Marmur procedure (7) as described previously (3). DNA was prepared from all other strains as described above except that cells were collected by centrifugation at 6,000 x g for 10 minutes.

PREPARATION OF PROBES

pTf8, pTf11, pTf32 and pTf34 were prepared from *T. ferrooxidans* ATCC 19859 as previously described (8). pTf64 was prepared from pTf8 by excision of the *Eco*RI — *Bgl* III fragment of the family I repeat and cloning of this fragment into Sp64 (9). pTf16 was prepared from pTf8 by excision of the *Bam*H1—*Eco*R1 fragment and cloning this fragment into pBR322. pTf20 was prepared from pTf8 by removal of the unique SphI fragment and reclosure of the residual plasmid. pTf22 was prepared from pTf32 by excision of the *Bam*HI — *Cla*I fragment and cloning of this fragment into pBR322.

Plasmid constructions were carried out by standard procedures (8). Recombinant plasmids were screened by a mini-procedure (10) and preparative amounts were obtained by the rapid heating method (11).

NICK-TRANSLATIONS

All nick translations were performed according to the manufacturer's instructions using a nick translation reagent kit (Bethesda Research Labs, Gaithersburg, MD) and 32p-labeled dCTP (800 Ci/mmol) (Amersham, Inc., Arlington Heights, IL). DNA was routinely labeled to greater than 10^8 cpm per microgram.

GEL ELECTROPHORESIS, SOUTHERN BLOTS AND HYBRIDIZATION

DNA was digested with restriction endonucleases (Bethesda Research Laboratories, Bethesda, MD (BRL) according to the manufacturer's recommendations. DNA fragments were separated in 0.8% agarose gels (BRL, Ultra Pure grade) in 0.04 M Tris (pH 8.0)-0.005 M Na acetate — 0.001 M EDTA.

Southern blots were carried out using GeneScreen™ Hybridization Transfer Membrane (New England Nuclear, Inc., Boston, MA) essentially according to the manufacturer's recommendations. Hybridization was done at Tm-32°C and the most stringent wash was done at Tm-37°C. Autoradiography was for different lengths of time on Kodak X-Omat X-ray film.

RESULTS

Presence of Family 1 and Family Repetitive DNA Sequences in Strains of T. ferrooxidans and T. thiooxidans

DNA probes were prepared containing either a family 1 repeat or a family 2 repeat as described in Materials and Methods. The DNA probes were used in Southern blot hybridization against the DNA of several strains of *T. ferrooxidans*, *T. thiooxidans*, several other Thiobacilli species and also several other bacterial species. Representative blots are shown in Fig. 1A (family 1 probe) and Fig. 1B (family 2 probe). A summary indicating the presence or absence of family 1 or family 2 repetitive sequences in various strains is given in Table I.

Of the 22 strains of *T. ferrooxidans* analyzed, about half (12/22) contained representative sequences of both families. One strain, ATCC 13661K, contained only family 1 and three strains, SHI G, SHIA and LL(TM), contained only family 2. The remaining six strains lacked both families (Table I).

In general, in those strains containing both families, there are more copies (10-20) of family 1 than family 2 repeats (5-10). ATCC 14119 is usual in that it contains only one copy of a family 2 repeat. Also, there appear to be significantly more copies of family 1 repeats (at least 40) in *T. thiooxidans* compared to *T. ferrooxidans*, although this number remains to be verified by other techniques in addition to Southern blotting.

The positions of several of the bands hybridized by family 2 appear to be conserved in many strains of *T. ferrooxidans* (Fig.1B) whereas in family 1, there does not appear to be such a high proportion of conserved bands, although some do exist (Fig. 1A).

Lastly, two independently derived colony isolates of *T. ferrooxidans* ATCC 13661 have strikingly different patterns of hybridization with family 1 and family 2 probes (Fig. 1B, B). ATCC 13661H was clonally purified in our laboratory from a stock obtained from the ATCC (American Type Culture Collection). ATCC 13661K was similarly isolated in the laboratory of Dr. C. Kulpa from the same stock obtained from ATCC. We propose that the ATCC stock of *T. ferrooxidans* ATCC 13661 contains at least two different populations of *T. ferrooxidans* with regard to its genomic arrangement of the repeated sequences.

POSITIONAL ANALYSIS OF FAMILY 1 AND FAMILY 2

Preliminary evidence has indicated that family 1 sequences are mobile and can move around the genome of *T. ferrooxidans* (5,12). We now present further evidence of family 1 mobility and extend these observations by demonstrating that family 2 is also mobile.

Evidence for mobility of family 1 within the genomes of several strains of *T. ferrooxidans* was obtained by Southern blot analysis using two types of probes. The first, exemplified by pTf64, contains DNA corresponding to only family 1 repeat. The second, exemplified by pTf8, contains DNA corresponding to a family 1 repeat with some additional flanking single copy DNA. A comparison of the hybridization of these two probes to a Southern blot of genomic DNA reveals whether a particular strain of *T. ferrooxidans* has a family 1 repeat located within a particular restriction fragment present in pTf8.

An example of such an experiment is shown in Fig. 2A. The probe pTf8 hybridizes to a 5.9kb *Eco*R1 restriction fragment in ATCC 23270 and to 4.3kb and 2.8kb *Eco*R1 restriction fragments in YS14. In contrast, the probe pTf64 does not hybridize to the 5.9kb fragment of ATCC 23270 nor to the 4.3kb fragment of YS14 indicating that these contain only the single copy DNA complimentary to that found in pTf8. The sum of the 2.8kb and 4.3kb bands in YS14 is 7.1kb which is 1.2kb larger that the 5.9 kb band of ATCC 23270. It is known that family 1 repeats contain an *Eco*R1 site and that the repeats are about 1.2kb in length (3). Therefore, the simplest model is that YS14 contains an insertion of a family 1 repeat carrying an endogenous *Eco*R1 site into a segment of single copy DNA in the position shown in Fig. 2A and that ATCC 23270 lacks the repeat in this position.

A similar experiment was carried out to evaluate positional changes in family 2 repeats (Fig. 2B). The probe pTf22 contains DNA corresponding to just the family 2 repeat whereas the probe pTf32 contains a family 2 repeat and additional flanking single copy DNA. Note that family 2 repeats do not contain an endogenous *Eco*R1 site. Using the same logic as presented above, there is evidence that

ATCC 19859 contains a family 2 repeat in the position indicated in Fig. 2B whereas strain Duval does not. Additional Southern blotting experiments (data not shown) confirm and extend these observations.

DEVELOPMENT OF INDUSTRIAL STRAINS

Preliminary evidence, obtained in collaborations with Drs. A. Agate of the MACS Institute, Pune, India and K. Natarajan of the Indian Institute of Science, Bangalore, India, has demonstrated the facility with which *T. ferrooxidans* ATCC 19859 can adapt to changing conditions. For example, more than 10 g/L of Cu^{+2} is normally toxic to this strain when growing in 9K-iron medium. However, by batch culturing with incremental increases in Cu^{+2} concentration *T. ferrooxidans* can be adapted to grow in 40g/L of Cu^{+2}.

We wished to exploit this proclivity for adaptation in order to generate a strain of *T. ferrooxidans* that would leach copper from an industrial silicone waste product produced by General Electric Silicone Products division at Waterford, NY.

The waste is generated during the production of silicones and consists of approximately 30% solids and 70% water. The major solid constituent is finely divided silica and minor constituents include copper (1-2%), calcium, iron and trace amounts of other metals including cadmium, lead and selenium. Currently, the waste is landfilled but changing government regulations may eliminate landfilling as an option for the disposal of the waste within a few years, jeopardizing the future of the Waterford facility. Therefore, identification of more productive uses of the solid waste is an economic and technological imperative. One of these opportunities is to recover the copper and use the residual solids in the manufacture of cement, concrete, masonary block or glass.

The laboratory of Dr. Henry Ehrlich has been evaluating the feasibility of using *T. ferrooxidans* ATCC 19859 to bioleach the copper from the silicone waste using either batch or continuous culturing of the waste with *T. ferrooxidans* in a medium supplemented with 9K-$FeSO_4$ and adjusted to pH2.5. We have analyzed the genomic locations of family 1 repeats before and after several months of subculturing of the *T. ferrooxidans* in batch culture with the waste materialusing cells provided by Dr. Ehrlich's laboratory. As shown in Fig. 3 there is a striking difference in the position of one of the repeats before and after the batch subculturing with the waste. We propose that this positional change is associated with the adaptation of the *T. ferrooxidans* to growth in media containing the waste. Other positional changes of family 1 have been observed with cells adapted to growth to normally toxic levels of Cu^{+2}. (data not shown) Clearly specific changes of the location of family 1 repeats within the genome of *T. ferrooxidans* are associated with the adaptation process, but additional experiments need to be carried to evaluate whether the changes are causing the adaptation or are a consequence of it.

There are several mechanisms that could explain how the repetitive DNA elements could be involved in adaptation. For example, they could promote intra-chromosomal recombination or plasmid-chromosome recombination, leading to new genetic combinations, some of which might provide the organism with a selective advantage in particular circumstances. Alternatively, the repetitive DNA elements could contain strong promoters or stop signals influencing the expression of nearby genes either positively or negatively.

The presence and mobility of repetitive DNA sequences in many strains of *T. ferrooxidans* from around the world suggests that they play an important role in promoting the survival of this species, although, as argued above, they cannot be essential since several strains lack them.

Repetitive DNA sequences have been found in a wide range of microorganisms including the archaebacterium *Halobacterium halobium* which has at least 50 different families of repeated sequences (20). Repeated sequences are also known in the gram-negative bacteria *Caulobacter crescentus* (13) and *Pseudomonas cepacia* (14), as well as in several species of the gram-positive genus *Streptomyces* (15-18), including one example in which amplified repeated sequences represent 45% of the genome (18).

The apparent genetic instability found within bacterial populations that contain repetitive elements might introduce a degree of genetic and phenotypic flexibility in response to changing environmental conditions. The ability of some members of a clonal population to survive under conditions that prevent growth of the majority of the population would enhance survival of the species.

Much work remains to be done on evaluating the importance, mechanism of action and causal relationship of repetitive DNA sequences to the genetic and phenotypic plasticity of *T. ferrooxidans*. There are also interesting questions to be addressed concerning the frequency and specificity of the genetic changes. For example, do the repetitive DNA sequences move at random around the genome of *T. ferrooxidans* or do they integrate into and excise from only particular regions of the genome, perhaps in a genetically programmed fashion? Can the rate of movement be influenced by external conditions including conditions known to induce physiological stress such as heat shock? Are there other classes of repetitive DNA elements in addition to family 1 and family 2 that remain to be discovered?

In addition to these fundamental issues we are also interested in further evaluating the adaptation of *T. ferrooxidans* to commercial metal recovery operations and in determining the role of mobile repetitive DNA sequences in the adaptation process. A key advantage of using such naturally adapted strains is that their use in a commercial operation does not come under the guidelines and regulations governing the use of genetically engineered strains.

ACKNOWLEDGEMENTS

We thank Thomas Clark and Dr. Henry Ehrlich for *T. ferrooxidans* cells grown in silicone waste. This work was partially supported by a grant from the National Science Foundation (MSM-8215220).

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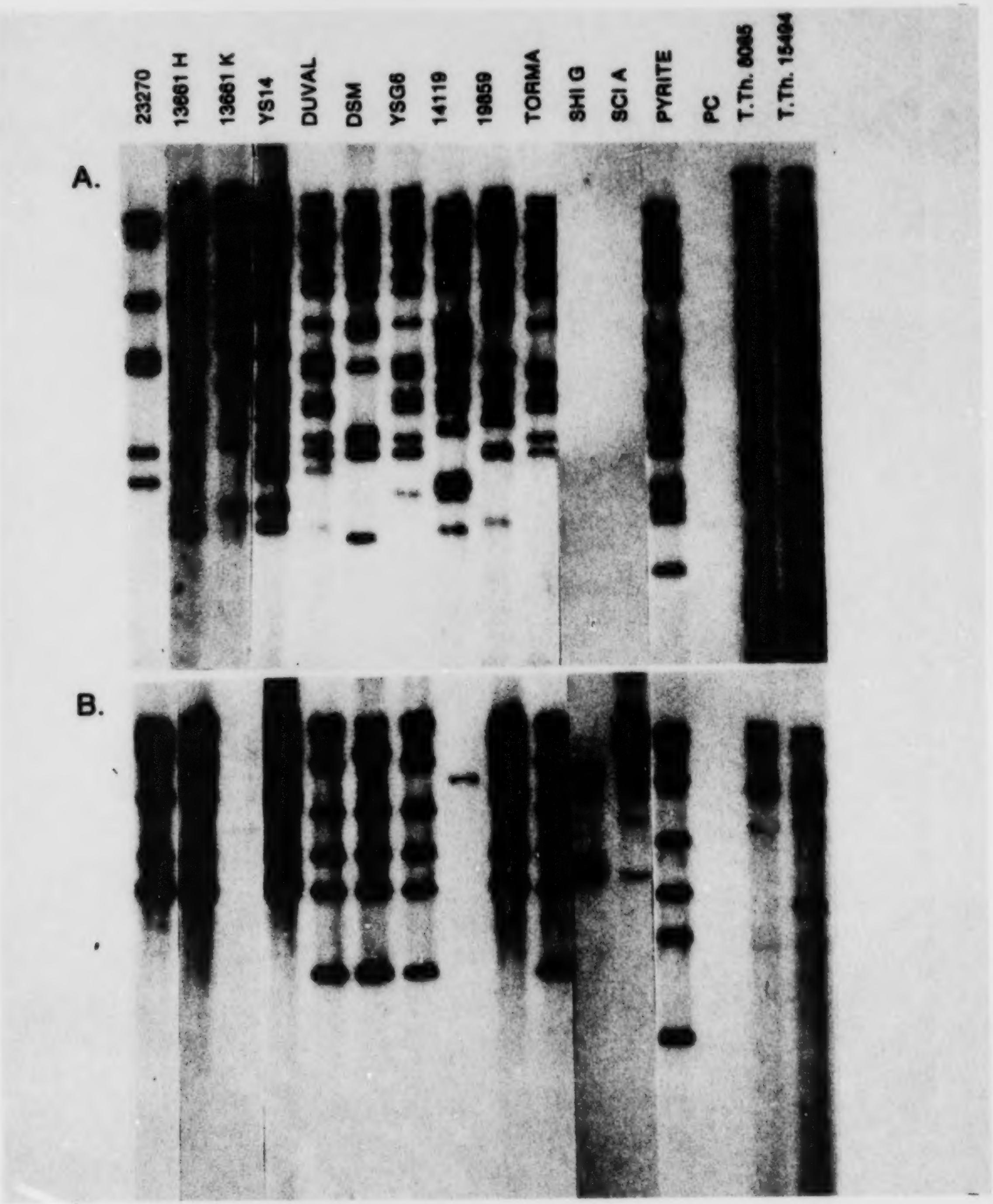
23270
13661 H
13661 K
YS14
DUVAL
DSM
YSG6
14119
19859
TORMA
SHI G
SCI A
PYRITE
PC
T.Th. 8085
T.Th. 15494
A.
B.

Fig. 1 Southern blot of *Eco*R1 cut DNA prepared from different strains of *T. ferrooxidans* and *T. thiooxidans*. (A) Probed with exclusively family 1 repeated DNA. (B) Probed with exclusively family 2 repeated DNA. The strain designations can be derived from inspection of the list of strains in Materials and Methods.

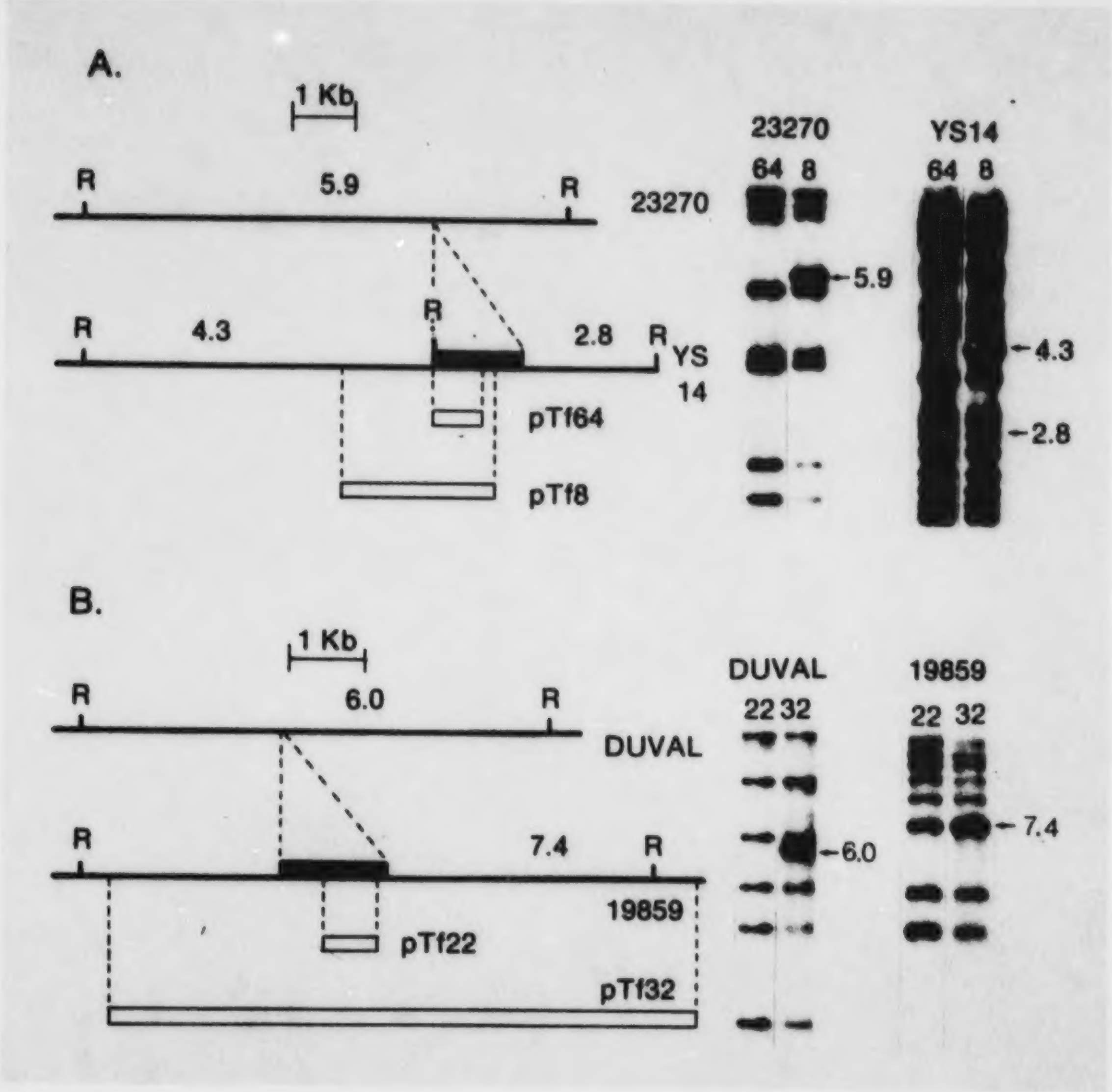
A.
1 Kb
R
5.9
R
23270
R
R
4.3
2.8
R
YS
14
pTf64
pTf8
23270
64 8
5.9
YS14
64 8
4.3
2.8
B.
1 Kb
R
6.0
R
DUVAL
R
7.4
R
19859
pTf22
pTf32
DUVAL
22 32
6.0
19859
22 32
7.4

Fig. 2 Detection of positional differences of (A) a family 1 repeat or (B) a family 2 repeat in several strains of *T. ferrooxidans*. Right hand side of the figure are Southern blots of genomic DNA from strains of *T. ferrooxidans* cut with *Eco*R1 and probed with either pTf8 ro pTf64 in (A) or pTf22 or PTf32 in (B). Left hand side of the figure are interpretations of the Southern blots described in the text. Numbers correspond to size in kb, R = *Eco*R1, = family 1 or family 2 repeat.

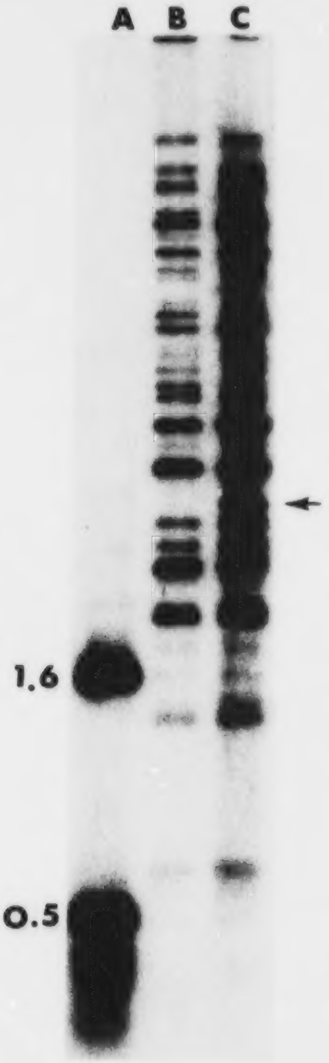

Fig. 3. Southern blot of DNA cut with *Bam*H1 and probed with a family 1 repeat. Lane A = BRL Ladder; Lane B = DNA from wild type *T. ferrooxidans* ATCC 19859 before exposure to solid waste; Lane C = DNA from the same strains as Lane B after 2 months batch culturing in the presence of solid waste. The arrow denotes a new band corresponding to a new position for a family 1 repeat. Sizes are in kilobases.

TABLE 1

DISTRIBUTION OF REPEATED DNA SEQUENCES IN STRAINS OF T. ferrooxidans AND T. Thiooxidans.

	Strain	Family 1	Family 1
T. ferrooxidans:			
A	19859	+	+
	13661H	+	+
	23270	+	+
	14119	+	+
	YS14	+	+
	YS14S	+	+
	TF203	+	+
	DUVAL	+	+
	DSM	+	+
	YSG6	+	+
	TORMA	+	+
	PYRITE	+	+
B	13661K	+	o
C	SHI G	o	+
	SCI A	o	+
	LL (TM)	o	+
D	SHI L	o	o
	SHI LS	o	o
	SHI H	o	o
	SHI J	o	o
	33020	o	o
	PC	o	o
T. thiooxidans:			
	19377	o	o
	8085	+	+
	15494	+	+

A = strains containing both family 1 and family 2;

B = strains containing just family 1;

C = strains containing just family 2 and

D = strains lacking both family 1 and family 2.

+ = Presence of repetitive DNA sequences.

o = Absence of repetitive DNA sequences. Strain designations can be deduced from the list provided in Materials and Methods. Data for the table was derived from Southern blots such as that shown in Figure 1 and other Southern blots not shown.

RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE (RuBisCO) IN THE THIOBACILLI

J.M. Shively, M.T. Stoner*, C.A. Williams*,*
R.S. English and R. Blake, II+*

ABSTRACT

The Calvin cycle is the primary path for utilization of carbon dioxide by the thiobacilli. The initial enzyme in this cycle is RuBisCO. We have examined *Thiobacillus denitrificans, Thiobacillus ferrooxidans, Thiobacillus intermedius, Thiobacillus neapolitanus* and *Thiobacillus thiooxidans* for different large subunit RuBisCO genes (rbcL) using heterologous gene hybridization and for different structural forms of RuBisCO by sucrose density gradient centrifugation. For hybridization studies, the DNAs were purified from lysed cells by cesium chloride-ethidium bromide density gradient centrifugation, digested with restriction endonucleases, electrophoresed in agarose gels, and blotted by the method of Southern. The *rbcL* of *Anacystis nidulans* (pANP1155) and *Rhodospirillum rubrum* (pRR116) were used as probes. Both probes gave positive results with *T. denifrificans*, *T. ferrooxidans*, *T. neapolitanus*, and *T. intermedius*. The positive restriction fragments were different for the two probes. This suggests that these thiobacilli possess two forms of RuBisCO. *T. thiooxidans* yielded positive results with only pANP1155. This implies the presence of only the hexadecameric RuBisCO. When supernatants from broken cells were subjected to sucrose density gradient centrifugation two structural forms of RuBisCO were noted only for *T. denitrificans*. An explanation for the discrepancy between DNA hybridization and enzyme characterization experiments with *T. ferrooxidans*, *T. neapolitanus*, and *T. intermedius* is under investigation.

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* Department of Biological Sciences Clemson University Clemson, SC

+ Department of Biochemistry Meharry Medical College Nashville, TN

LA RIBULOSE BISPHOSPHATE CARBOXYLASE / OXYGÉNASE (RuBisCO) CHEZ LES THIOBACILLES

J.M. Shively, M.T. Stoner*, C.A. Williams*, R.S. English* et R. Blake II+*

RÉSUMÉ

Le cycle de Calvin est la voie principale d'utilisation du gaz carbonique par les thiobacilles. La première enzyme impliquée dans ce cycle est la RuBisCo. Nous avons examiné *Thiobacillus denitrificans, Thiobacillus ferrooxidans, Thiobacillus intermedius, Thiobacillus neapolitanus* et *Thiobacillus thiooxidans* pour différents gènes (rbcL) à larges sous-unités codant pour la RuBisCO par utilisation d'hybridation de gènes hétérologues et pour différentes formes structurales de la RuBisCo par centrifugation par gradient de densité de sucrose. Pour les études d'hybridation, les ADN. ont été purifées de cellules lysées à l'aide de centrifugation par gradient de densité de chlorure de césium et de bromure d'éthidium. Ensuite, ils ont été digerés par des endonucléases de restriction, séparés par électrophorèse en gel d'agarose et finalement analysés par la méthode de Southern. Le gène rbcL *d'Anacytis nidulans* (pANPI 155) et de *Rhodospirillum rubrum* (pRR 116) ont été utilisés comme sonde. Ces deux sondes ont donné des résultats positifs avec *T. denitrificans, T. ferrooxidans, T. neapolitanus* et *T. intermedius.* Les fragments de restriction obtenus étaient différents des deux sondes, ceci suggère que ces thiobacilles possèdent deux formes de l'enzyme RuBisCO. *T. thiooxidans* a donné des résultats positifs qu'avec pANP1155, cela implique la présence unique d'une RuBisCO hexadécamérique. Lorsque les surnageants des cellules brisés de *T. denitrificans* ont été soumis à la centrifugation par gradient de densité de sucrose, deux formes structurales de la RuBisCO ont été observées seulement chez cette souche. Une explication de la divergence entre les expériences hydridation de l'ADN. et de la caractérisation par enzymes chez *T. ferrooxidans, T. neapolitanus* et *T. intermedius* est présentement à l'étude.

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* Département des Sciences Biologiques Clemson Université Clemson, SC É.-U.

+ Département de Biochimie, Collège Médical Meharry Nashville, TN É.-U.

INTRODUCTION

The thiobacilli can utilize carbon dioxide to satisfy their total cellular carbon requirements. They sequester the carbon dioxide via a complex series of reactions referred to as the Calvin cycle; the same cycle utilized by algae and higher plants. The enzyme responsible for the actual fixation of carbon dioxide, RuBisCO, can occur in at least three different structural forms (Tabita, 1988). The most common form which is present in nearly all carbon dioxide-fixing organisms, is a hexadecamer (Form I) composed of eight large (LSU) and eight small (SSU) subunits. The LSU contains the catalytic site. RuBisCO (Form II) with only LSU have been reported in *Rhodospirillum rubrum* and *Rhodobacter* species (Gibson and Tabita, 1977; Shively, *et al.*, 1984; Tabita, 1988). The *R. rubrum* enzyme is a dimer of LSU. The quaternary structure of the *Rodobacter* Form II RuBisCO is still in doubt, but it appears to be either a tetramer or hexamer of LSU. *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* possess both a Form I and Form II RuBisCO (Gibson and Tabita, 1977; Shively, *et al.*, 1984). The sequence of the gene (*rbc*L) for the LSU of the Form I enzyme is highly conserved; greater than 70% sequence homology between genes is common (Shively, *et al.*, 1984; Tabita, 1988). The sequence of the genes for the Form II enzymes are also similar, but are very dissimilar to the *rbc*L of Form I (Wagner, *et al.*, 1988). Herein we report examination of several thiobacilli for different structural forms of RuBisCO using heterlogous gene hybridization and sucrose density gradient centrifugation.

MATERIALS AND METHODS

Organisms and Sources. The *Thiobacillus* species examined were *T. denitrificans* ATCC 25259 (R. Blake, II., Meharry Medical College, Nashville), *T. ferrooxidans* ATCC 23270, A6, and 11 Fe (T. Ward, INEL, Idaho Falls, ID), *T. intermedius* K12 and D14 (E. Bock, Institut Allgemeine Botanik, University of Hamburg, Hamburg, FRG), *T. neapolitanus* (G. Kuenen, Delft Technical University, Delft. The Netherlands), and *T . thiooxidans* ATCC 8085 (T. Ward, INEL, Idaho Falls, ID). *R. capsulatus* PAS100 was obtained from B. Marrs, E.I. DuPont de Nemours and Co., Wilmington, DE.

Methods of Cultivation. *T. denitrificans* was grown anaerobically in screw-cap bottles with nitrate as the electron acceptor (Shively, *et al.*, 1970). *T. ferrooxidans* was cultured in the medium of Tuovinen and Kelly (1973). *T. intermedius* and *T. neapolitanus* were grown in the medium of Vishniac and Santer (1957). *T. thiooxidans* was grown with elemental sulfur as the energy source (Barton and Shively, 1968). *R. capsulatus* was cultured anaerobically under limiting carbon dioxide as previously described (Shively, *et al.*, 1984). All of the organisms were grown as batch cultures to the late logarithmic growth phase and harvested by centrifugation at 4°C. The cells were resuspended and centrifuged 2-4 times in 10mM Tris-HCl-1mM EDTA buffer (pH 8.0). For enzyme preparations the cells were used immediately. For DNA purification the cells were frozen as pellets at -70°C until needed.

RuBisCO Experiments. All purification procedures were accomplished at 4°C. The cells were resuspended in Tris-HCl, 10mM; EDTA, 1mM; $MgCl_2$, 15mM; $NaHCO_3$, 2mM; DTE, 1mM; pH 8.0, passed 3x through a French pressure cell at 20,000 psi, and centrifuged at 48,000 xg for 1 hr. The supernatants were subjected to sucrose density gradient centrifugation (Shively, *et al.*, 1984). The gradients were fractionated and the fractions assayed for RuBisCO as previously described (Shively *et al.*, 1973, 1984).

DNA Purification and Analysis. The frozen cell pellets were thawed and the cells resuspended in 50mM Tris-HCl buffer (pH 8.0) containing 25% (w/v) sucrose. Lysis was accomplished by EDTA-lysozyme-detergent and the DNA isolated by cesium chloride-ethidium bromide density gradient centrifugation (Shively, *et al.*, 1986). Restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting, probe preparation and heterologous hybridization were accomplished as described by Shively, *et al.* (1986). The probes used in these experiments were the rbcL of *Anacystis nidulans* (1.5 kbp *EcoRI -PstI* fragment from pANP1155 encomposing nearly the total gene) and *Rhodospirillum rubrum* (two internal *SalI* gene fragments encomposing about one-third of the RuBisCO gene subcloned into pBR322 from pRR116). With our stringent hybridization conditions the pANP and pRR probes do not cross hybridize.

Hybridization with the pANP probe implies the presence of a Form I RuBisCO and with the pRR probe a Form II enzyme.

RESULTS

Sucrose density gradient centrifugation resulted in two peaks of RuBisCO activity with *R. capsulatus* and *T. denitrificans* (Fig. 1). Previous experimentation (Shively, *et al*., 1984) demonstrated the presence of both a Form I and Form II RuBisCO in *R. capsulatus*. This data supports the contention that two forms of RuBisCO are also present in *T. denitrificans*. Only one peak activity corresponding to Form I RuBisCO was seen with *T. thiooxidans* (data not shown) and *T. ferrooxidans* (Fig. 2). A small amount of activity was noted in *T. ferrooxidans* corresponding to Form II RuBisCO. Preliminary electrophoretic evidence suggests that this might be a second form of RuBisCO.

Positive hybridization was obtained with the DNA of all of the thiobacilli using the pANP probe (Fig. 3). Several different endonuclease digests have been utilized, but only *EcoRI* disests are shown. With the exception of *T. thiooxidans*, all of the thiobacilli also gave positive hybridization with the pRR probe. In all cases the restriction fragments were different from those obtained with the pANP probe.

We originally reported that *T. intermedius* K12 DNA did not show positive hybridization with the pRR probe (Shively, *et al*., 1986). However, when the DNA of the D14 strain of *T. intermedius* gave positive results we repeated the experiment with T. intermedius K12 using a pRR probe of higher specific radioactivity; a weak. but definitely positive signal was obtained (Fig. 3).

DISCUSSION

A positive hybridization signal with both probes suggested the presence of two structural forms of RuBisCO in all of the thiobacilli tested except *T. thiooxidans* . One of the forms would be the common Form I enzyme composed of 8LSU and 8SSU and the other would be similar to either the Form II enzyme of *R. rubrum* or *Rhodobacter* species.

The sucrose density gradient experimentation showed two structural forms of RuBisCO in *T. denitrificans*. McFadden and Denend (1972) reported a RuBisCO in *T. denitrificans* with a molecular weight of 350,000; a Form II structure is inferred. Our hybridization and sucrose gradient data supported this observation, but also demonstrated the presence of a Form I enzyme.

A slight shoulder on the upper side of the Form I RuBisCO peak in the sucrose gradient of *T. ferrooxidans* aligned close to the Form II enzyme peak of *R. capsulatus*. Preliminary electrophoretic evidence suggests that this might be a second form of RuBisCO. Holuigue, *et al*. (1987) reported the RuBisCO of *T. ferrooxidans* to be a hexadecamer with a native M_r of 570,000. They also showed the presence of two other forms of RuBisCO in *T. ferrooxidans* with M_r of 330,000 and 115,000. They attributed the occurrence of these two forms to the dissociation of the hexadecamer. The subunit composition and the concentration of the two forms was not given. One could hypothesize that they represent the product(s) of a different gene(s).

The hybridization and sucrose gradient data are in complete agreement for *T. denitrificans*, *T. thiooxidans*, and possibly *T . ferrooxidans*, however only Form I RuBisCO has been demonstrated in *T. intermedius* (Bowman and Chollet, 1980) and *T. neapolitanus* (Snead and Shively, 1978). Since the pRR probe gave positive results one would also expect a Form II enzyme; this discrepancy needs clarificaiton. It is of interest that *Thiobacillus novellus* (McCarthy and Charles, 1975) and *Thiobacillus versutus* (Charles and White, 1987) apparently also produce only a Form I enzyme; hybridization data is not available. With the exception of *T. denitrificans* the hybridization with the pRR probe may represent an artifact, i.e. the hybridization is not to a RuBisCO gene, but to a similar segment in a different gene. This seems unlikely since we are using stringent hybridization conditions. It seems more plausible that the Form II gene is under tight regulatory control. We are investigating conditions for the expression of the different RuBisCO genes as well as their cloning and expression in *Escherichia coli.*

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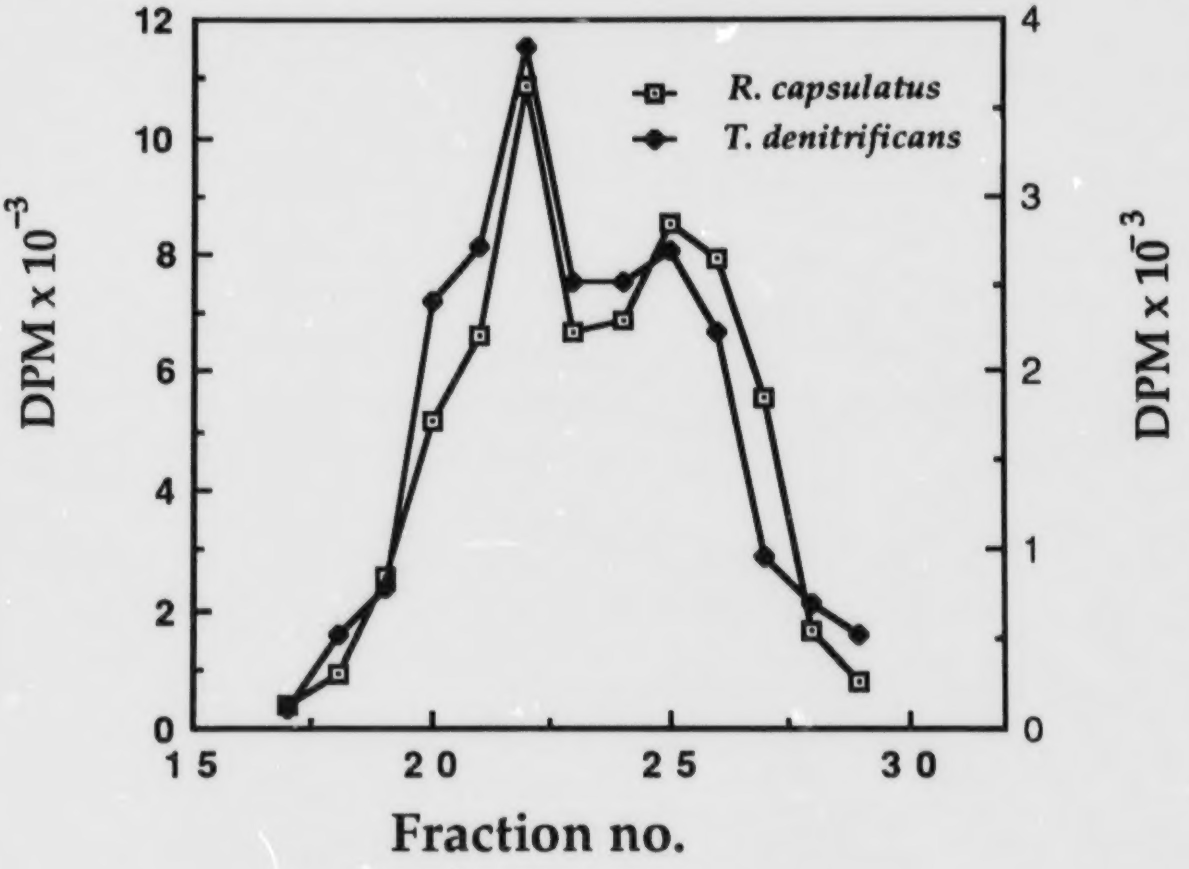

Fig. 1 RuBisCO activity in fractions from sucrose density gradients of cell supernatants of *T. denitrificans* and *R. capsulatus*. The top of each gradient is on the left. The peak to the right is Form I RuBisCO. The peak to the left is Form II. The x-axis for *R. capsulatus* is on the right and for *T. denitrificans* on the left.

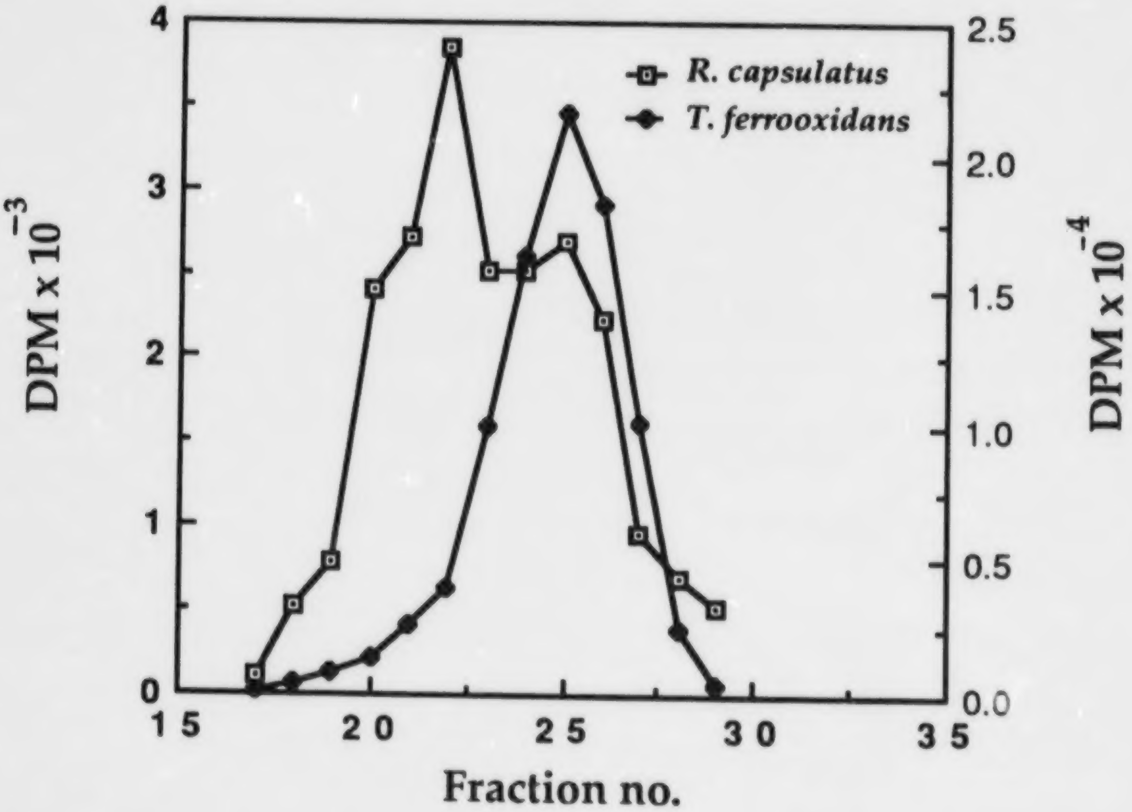

Fig. 2 RuBisCO activity in fractions of sucrose density gradients of cell supernatants of *T. ferrooxidans* ATCC 23270 and *R. capsulatus*. The top of each gradient is on the left. For *R. capsulatus* the peak to the right is the Form I RuBisCO and the one to the left is the Form II. The x-axis for *R. capsulatus* is on the left and for *T. ferrooxidans* on the right.

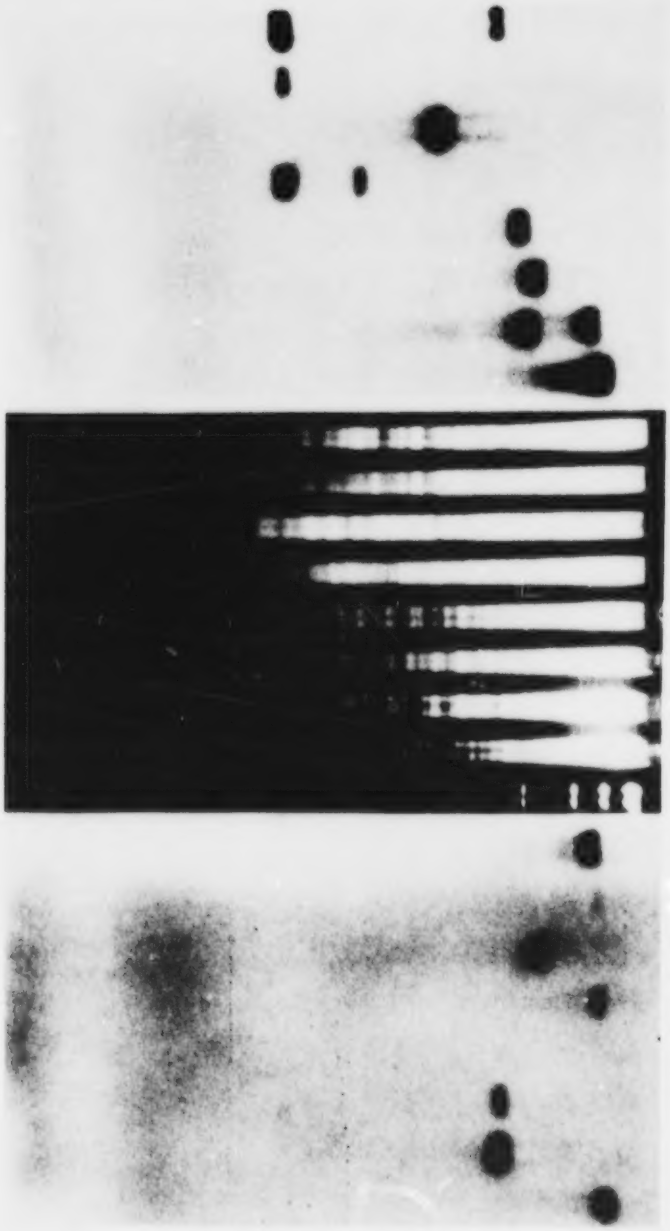

Fig. 3 Hybridization of the *A. nidulans* (pANP) and *R. rubrum* (pRR) probes to the DNA of the *thiobacilli*. Middle panel: Ethidium bromide stained agarose gel. The two gels for the blots were identical. Left hand panel: Autoradiogram of Southern blot hybridized with pANP probe. Right hand panel: Autoradiogram of Southern blot hybridized with pRR probe. All *Thiobacillus* DNAs were digested with *EcoRI*. A *Hind III*digest of l DNA is shown in the far right lane of the middle panel. *Thiobacilli* from left to right; *T. intermedius* D14, *T. intermedius* K12, *T. denitrificans*, *T. neapolitanus*, *T. thiooxidans*, *T. ferrooxidans* ATCC 23270, *T. ferroxidans* 11 Fe, and *T. ferrooxidans* A6.

INTRODUCTION AND REPLICATION of BROAD-HOST RANGE, RP4-BASED PLASMIDS IN ACIDOPHILIC BACTERIA.

F. F. Roberto, A. W. Glenn and T.E. Ward,

Biotechnology, Idaho National Engineering Laboratory,
Idaho Falls, ID 83415-2203 USA

ABSTRACT

Acidophilic bacteria, including *Thiobacillus ferrooxidans* and members of the genus *Acidiphilium*, are readily isolated from acidic mine environments and are among the principal agents of mineral leaching.

The genetic characteristics of these bacteria are being examined in our laboratory to allow them to be used in large scale/industrial processes. Genetic manipulation of acidophiles to introduce desirable characteristics, which might include heavy metal resistance and capability for rapid growth on simple nutritional substrates, requires the development of methods for the introduction of foreign genes, as well as efficient expression and stable maintenance of the introduced genetic information. To this end, we have recently introduced two broad-host range cloning vectors, pRK415 and pLAFR3, as well as a number of pBR328/*Acidiphilium* chimeric plasmids into *Acidiphilium facilis* by two distinct methodologies.

These plasmids have been efficiently mobilized to *A. facilis* from an *E. coli* donor. They have also been introduced as naked DNA molecules by electroporation. In both cases, the plasmids have been detected by Southern blot hybridization, and high-level expression of the tetracycline and chloramphenicol anti biotic resistance genes carried by these plasmids has been observed.

Experiments are in progress to further refine this transformation system and extend its range to *Thiobacillus* species.

INTRODUCTION ET RÉPLICATION DE PLASMIDES À LARGE SPECTRE D'INFECTIVITÉ CONTENANT RP4 CHEZ DES BACTÉRIES ACIDOPHILES

F.F. Roberto, A.W. Glenn et T.E.Ward

Biotechnologie, Idaho National Engineering Laboratory
Idaho Falls, ID 83415-2203 É.-U.

RÉSUMÉ

Des bactéries acidophiles, comprenant *Thiobacillus ferrooxidans* et des membres du genre *Acidiphilium*, sont facilement isolées à partir d'environnements miniers acides et comptent parmi les principales causes de la lixiviation de minéraux.

Les caractéristiques génétiques de ces bactéries sont présentement à l'étude dans notre laboratoire afin de pouvoir s'en servir dans des procédés industriels à grande échelle. Pour pouvoir effectuer des manipulations génétiques en vue d'introduire chez ces bactéries acidophiles des caractéristiques désirables comme la résistance aux métaux lourds et la capacité de se développer rapidement à partir de substrats simples, des méthodes pour introduire des gènes étrangers, s'assurer qu'ils s'expriment de façon efficace et que l'information nouvellement introduite soit stable, sont nécessaires. À cette fin, nous avons récemment utilisé deux méthodes de clônage pour introduire deux vecteurs à large spectre d'infectivité, pRK415 et pLAFR3, ainsi qu'un certain nombre de plasmides chimères pBR328/*Acidophilium* dans *Acidophilium facilis*.

Ces plasmides ont été mobilisés chez *A. facilis* à partir d'une souche donatrice de *E. coli*. Ils ont aussi été introduits seuls comme molécules d'ADN par électroporation. Dans les deux cas, les plasmides ont été détectés par hybridation de Southern et une forte expression des gènes de résistance aux antibiotiques tétracycline et chloramphénicol portés par ces plasmides, a été obtenue.

D'autres études sont en cours pour améliorer ce système de transformation et élargir le spectre d'infectivité aux espèces de *Thiobacillus*.

INTRODUCTION

Acidophilic bacteria, including members of the genera *Thiobacillus*, and *Acidiphilium*, are readily isolated from acidic mine drainage. *Thiobacillus ferrooxidans* has been shown to be a major agent in the production of acidic effluents in mining environments, which is a result of its action on mineral sulfides. There is evidence to suggest that *Acidiphilium*, a heterotrophic bacterium, promotes the activity of *T. ferrooxidans*, perhaps by scavenging organic by-products which are toxic to *T. ferrooxidans* (B. Johnson, pers. commun., 1989).

The mining industry currently exploits the capacity of *T. ferrooxidans* to solubilize metals from ore in the production of copper, gold and uranium, and there is considerable interest in extending its use to other areas, including the depyritization of coal.

Considerable effort is being devoted to developing techniques for the genetic manipulation of *T. ferrooxidans* (Davidson and Summers, 1983; Holmes, *et al.*, 1984; Rawlings and Woods, 1985, 1988; Rawlings, *et al.*, 1984,1986). The ability to introduce novel traits, such as metal resistances or improved growth characteristics, would be of benefit to commercial use of the bacterium in bioleaching processes.

Plasmids have been isolated from a number of strains of *T. ferrooxidans*, which could prove useful in the construction of vectors to carry exogenous genetic information into the bacterium. A plasmid from *T. ferrooxidans* strain FC has been shown to possess a broad-host range origin of replication with considerable homology to the origin of the incQ plasmid, RSF1010 (Dorrington and Rawlings, 1989). We are attempting to introduce an RP4-based replicon (incP-1) into *T. ferrooxidans*, first refining the methodology in a heterotrophic acidophile, *Acidiphilium facilis*. It should be possible to extend the transformation protocol we develop in *A. facilis* to *T. ferrooxidans*, or, alternatively, directly transfer plasmids by conjugation. This paper describes our efforts to date.

MATERIALS AND METHODS

Bacterial strains. *Acidiphilium facilis* type strain PW2 was acquired as ATCC strain 35904. Other *Acidiphilium* strains used, CM3A, CM5, CM9, and CM9A were isolated from water collected at the Blackbird cobalt mine, 20 miles southwest of Salmon, Idaho (D. Thompson and P. Wichlacz, unpub. observ.), while PW1 from acidic mine drainage sites in central Pennsylvania was obtained from P.L. Wichlacz (Wichlacz and Unz, 1981). Escherichia coli strain S17.1 (*thi*, *pro*, *hsd*R^-, *hsd*M^+, *rec*A, RP4-2-Tc::Mu-Km::Tn7; Simon, *et al.* ,1983) used in conjugation experiments was obtained from T. Kosuge.

Bacterial media. *Acidiphilium* strains were grown in Modified Acidophile Salts (MAS) media, which is a modification of that used by Wichlacz and Unz (1981). MAS medium contained 1 mM $(NH_4)_2SO_4$, 2 mM KCl, 0.86 mM K_2HPO_4, 10 mM $MgSO_4$, 6.6 mM $CaCl_2$, 2.6 mM $FeSO_4$, 0.01% yeast extract and 0.1% glycerol, pH 3.5. Solidified media were prepared with the addition of 0.5 % GelRite gellan gum (Kelco) or 1% agarose (Sigma # A-6013). Where indicated, tetracycline (Tc) or chloramphenicol (Cm) was added to the media at 40 μg/ml and 70 μg/ml, respectively.

Plasmids. Plasmids used in this study included the RP-4 based vectors, pRK415 (Keen, *et al.*, 1988) and pLAFR3 (Staskawicz, *et al.*, 1987), which carry tetracycline resistance markers. Also used in electroporation experiments were a number of chimeric plasmids constructed from pBR328 (Soberon, *et al.*, 1980) and a variety of endogenous, cryptic plasmids from *Acidiphilium* species. Plasmids pL13-4, pL15-2 and pL19-4 contained *Acidiphilium* plasmids cloned into the EcoRI site of pBR328 (Cm gene location) and conferred Tc resistance. Plasmids pL14-2, pL18-2 and pL20-3 contained *Acidiphilium* plasmids cloned into the BamHI site (Tc gene location) and conferred Cm resistance. The *Acidiphilium* species from which the *Acidiphilium* component of these plasmids were derived is listed (in parentheses) in Table 3c. Plasmid pBR328 was obtained from Boehringer Mannheim.

Isolation of plasmids from *Acidiphilium* strains. Plasmids were isolated using the alkaline lysis method of Birnboim and Doly (1979), as described for small scale isolations in Maniatis (1982).

Restriction enzyme digestions, agarose gel electrophoresis and Southern blots. Restriction enzymes were purchased from Boehringer Mannheim and Promega, and digestions were carried out in appropriate buffers according to the manufacturers' instructions. Agarose gel electrophoresis was typically performed using 0.4% agarose (FMC, LE grade) gels prepared in 0.5X TBE and run in the same buffer (Maniatis, 1982). After running, gels were stained in a 2 μg/ml ethidium bromide solution for 30 minutes and DNA was visualized with a UV transilluminator (Spectroline) at 310 nm. Gels were prepared for transfer to Nytran (Schleicher and Schuell) or Biotrace RP (Gelman) by the alkaline transfer method (Reed and Mann, 1985). Blots were hybridized with digoxigenin-labeled pRK41 and developed according to manufacturer's instructions (Boehringer Mannheim, Genius non-radioactive detection kit).

Conjugal transfer from E. coli to *Acidiphilium*. Spot matings were performed essentially as described by Miller (1972). One milliliter exponential cultures of donor and recipient cells were centrifuged, washed twice and resuspended in 1 ml of 1 mM HEPES, pH 7.0. Donor cells were diluted 50-fold and a 25 μl aliquot was spotted onto a dry nutrient agar (Difco) plate. The liquid was allowed to absorb into the agar, at which time a 25 μl aliquot of the undiluted recipient cells was spotted directly onto the dried donor cells. Matings were allowed to proceed for three hours, after which the cells were recovered and resuspended in 200 μl MAS medium. The cells were then plated on solid MAS medium containing 40 μl/ml Tc. Transconjugants usually appeared within 3-4 days.

Electroporation of *Acidiphilium* A BTX Transfector 100 electroporation device was used. Late log phase cells were washed and concentrated to cell densities of between 10^9-10^{11} cells/ml in 1 mM HEPES, pH 7.0. Pulse durations and field strengths were varied as reported. After electroporation, cells were diluted 20-fold into MAS medium to allow expression of the antibiotic resistance phenotype. Where Tc selection was used, 1 μg/ml Tc was added to expression medium to induce the Tc resistance genes. Cell numbers approximately doubled during the 17-20 hour expression period. Cells were plated on 40 μg/ml Tc or 70 μg/ml chloramphenicol in MAS medium, and transformed colonies were clearly visible after three days. The experiments were performed at 32°C unless otherwise indicated.

RESULTS

Transfer of pRK415 to *Acidiphilium* from *E. coli*. The plasmid pRK415 was transferred to *A. facilis* from *E. coli* S17.1 by conjugation. *E. coli* strain S17.1 contains an integrated copy of RP4, and thus, directs the production of transfer functions which allow mobilization of plasmids carrying an origin of conjugal transfer (*ori*T) and a *mob* locus, such as pRK415 and pLAFR3. We first determined the tolerance of *A. facilis* for neutral pH conditions under which the matings would take place. Strain PW2 demonstrated a half-life at pH 7.0 on nutrient agar of 2 hours, while that for CM9A was 20 hours (data not shown). PW1 was not tested for this characteristic. An interesting feature of the transfer efficiency ,of pRK415 to *Acidiphilium* is its apparent strain dependence (Table 1). Nearly a thousand-fold greater efficiency is seen when comparing conjugation into strain PW1 with that seen with strain CM9A. PW2 showed an in termediate frequency.

DNA hybridization analysis. The presence of pRK415 and pLAFR3 in plasmid DNA preparations of putative PW2 transformants was confirmed by Southern blot an alysis (Figure 1). Lane 1 contained DNA from the untransformed strain, while lane 2 contained DNA isolated from PW2 electroporated with pLAFR3, lanes 3-9 were preparations of individual colony isolates from electroporations of PW2 with pRK415, and the authentic plasmid, pRK415, was run in lane 10. All plasmid preparations were digested with EcoRI prior to electrophoresis and transfer to BioTrace RP. Hybridization of labelled pRK415 was observed with pLAFR3 as both plasmids are derived from pRK290 (Ditta, *et al.*, 1985). Similar results were observed upon analysis of plasmid DNA isolated from CM9A transconjugants (data not shown).

Electroporation of *Acidiphilium*. We next attempted to transform strain PW1 directly with pRK415 DNA by electroporation. Bacterial electroporation has been shown to require high electrical field strengths when compared to electroporation of mammalian and plant cells. Our results demonstrate this requirement, with optimal transformation frequencies observed at a field strength of 15 kV/cm

(Figure 2), regardless of the pulse duration (5 or 10 ms exponential pulse). Transformation decreased above this field strength, presumably due to increasing cell death, which has been observed at high field strengths by other researchers. Increasing the DNA concentration in the incubation mixture results in increasing numbers of transformants (Figure 3). In data not shown, DNA concentrations have been increased to as high as 10 μg/ml and the number of transformants has been observed to increase even more.

Other factors influencing transformation by electroporation. The recipient strain used was observed to have a significant impact on the transformation efficiency by electroporation, and is reminiscent of the ability of the various strains to accept DNA by conjugation (Table 3a). About 1 transformant/μg pRK4 15 DNA was recovered from electroporations of strain CM3A, while some 2000 transformants per μg were obtained with strain PW1. This strain dependency may reflect an inherent difference in the physical characteristics of the cell membranes among strains.

The temperature at which the bacteria were grown prior to electroporation was also observed to have a significant effect on transformation efficiency (Table 3b). In strain CM9A, no transformants were recovered when cells were grown at 21°C, while a ten-fold increase was seen upon growth between 32°C and 37°C. Twenty-seven transformants/μg pRK415 were seen in strain PW1 grown at 21°C, with roughly one hundred-fold more found after growth at 32°C and a further ten-fold increase when the growth temperature was raised to 37°C. Culture temperature might be expected to alter the membrane lipid composition, as well as the fluidity of the membrane, which may explain this result. Such changes could allow greater influx of exogenous DNA.

Introduction of chimeric plasmids by electroporation. Cryptic plasmids from *Acidiphilium* were cloned into either the BamHI site (Tc gene) or EcoRI site (Cm gene) of the cloning vector, pBR328. A description of some salient features of the *Acidiphilium* plasmids, including restriction sites and size, is found in Table 2. Constructs derived from pCM4A-1 (pL13-4, pL14-2), pCM3A-1 (pL18-2), pBBW (pL15-2) and pCM4-2 (pL19-4, pL20-3) were tested in electroporation experiments with strain PW1. The results of electroporation with 2.5 μg/ml of each of these plasmids is shown in Table 3c. *Acidiphilium* plasmids from strains CM4A-1, BBW and CM4-2, cloned into the Cm gene of pBR328 and selected for on Tc, were all maintained in strain PW1. In contrast, only pL20-3 (pCM4-2 cloned as an BamHI fragment in the Tc gene) was maintained on Cm selection. This suggests that in pL14-2, disruption of the native plasmid at the BamHI si te somehow disrupts an essential function, perhaps the origin of replication. It is also important to note that pBR328 alone was unable to transform PW1 to either Tc or Cm resistance, suggesting that the ColE1 origin of replication (ori) is not recognized by the *Acidiphilium* replicative machinery. Transformation by the chimeric plasmids is therefore probably due to the functioning of the native *Acidiphilium ori*. Plasmid pRK415 was included in the experiment as a positive control, since we had already demonstrated that the broad-host range plasmid functioned in *Acidiphilium*.

CONCLUSIONS

Acidiphilium strains have been transformed by conjugation with *E. coli*. In order to accomplish this, suitable conditions allowing mating to occur (neutral Electroporation has also been demonstrated to be an efficient means to transform *Acidiphilium*.

The recipient strain and the growth temperature at which the bacteria are grown prior to electroporation, have been shown to have a profound effect on the transformation efficiency. The effect of growth temperature may be at the level of the cell membrane lipid composition. Influence of the strain selected on transformation efficiency has been observed in other microorganisms, including *E. coli* (Jessee, *et al.*, 1989). ColE1-based replicons apparently do not replicate in *Acidiphilium*, as shown by the failure to recover pBR328-transformed PW1 cells. Electroporation with chimeric recombinant plasmids containing entire *Acidiphilium* plasmids in pBR328 did yield transformants, as might be expected, since the *Acidiphilium*-derived portions of these plasmids no doubt contain origins of replication recognized by PW1.

The ability to insert foreign DNA into acidophilic bacteria will allow a variety of genetic manipulations to be performed which should result in the introduction of useful traits, and thus, the improvement, of these bacteria.

It is also hoped that the extension of these methodologies to *Thiobacillus ferrooxidans* will allow study and manipulation of gene expression in this important microorganism.

ACKNOWLEDGEMENTS

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Table 1

Transfer of plasmids to *Acidiphilium* by conjugation with *E. coli*.

Strain	Plasmid	Mating frequency, per recipient[a]
CM9A	pRK415	3×10^{-8}
PW2	"	1.67×10^{-6}
PW1	"	9.86×10^{-6}

[a] Transconjugant frequencies obtained from matings with 10^8 recipients
Matings were performed for 3 h on pH 7 media

Table 2

Endogenous plasmids in *Acidiphilium* strains.

Plasmid	Size, kb	Restriction enzyme sites[a,b]
PLHet	7.9	E, H(2), B, X(3), Bg, S, Sp
pCM3A	2.8	E, Bg
pBBW	3.9	E, Sp
pCM4-1	1.5	none
pCM4-2	4.4	E, H(2), Bg, P, V, K
pCM4-3	6.9	E, Bg
pCM4A-1	2.8	e, Bg
pCM4A-2	6.6	E, X, v(3), C,

[a] Abbreviations: E-EcoRI; H–HindIII; X–XhoI; Bg–BglII; S–SalI; Sp–SphI; P–PstI; V–EcoRV; C–ClaI; K-KpnI; none–dit not digest with enzymes tested

[b] Unless otherwise indicated (number in paratheses), only one site present

Table 3

Factors Influencing Transformation of *Acidiphilium* by Electroporation

A. Effect of recipient strain (pRK415)

Strain	Transformants/μg DNA
CM3A	0.8
CM5	2.6 x 10^2
CM9	1.9 x 10^2
CM9A	3.2
BBW	1.8 x 10^1
PW1	2.1 x 10^3
PW2	4.8 x 10^2

B. Effect of growth temperature (pRK415)

	Transformants/μg DNA		
Strain	21°C	32°C	37°C
CM9A	0	3.2	3.2 x 10^1
PW1	2.7 x 10^1	1.9 x 10^3	2.3 x 10^4

C. Effect of different plasmid constructs (PW1, 37°C)

Selection	Plasmid	Transformants/μg DNA
TC	pL13-4 (CM4A-1)	1.8 x 10^4
	pL15-2 (BBW)	8.8 x 10^4
	pL19-4 (CM4-2)	1.5 x 10^4
	pRK415 (RK2)	5.1 x 10^4
Cm	pL14-2 (CM4A-1)	0
	pL18-2 (CM3A-1)	0
	pL20-3 (CM4-2)	1.2 x 10^4
Tc, cm	pBR328	0

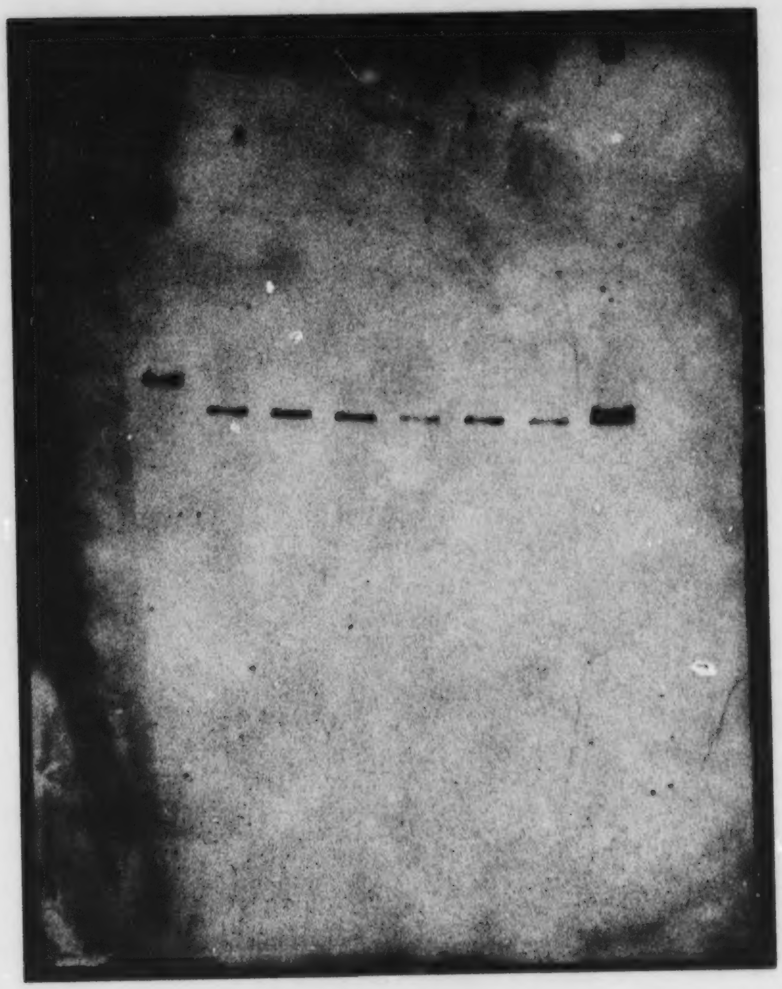

Fig. 1 Southern blot analysis of PW2 transconjugants. Plasmids from conjugation of PW2 and *E. coli* were blotted to a nylon membrane and the blot was hybridized with digoxigenin-labelled pRK415 as described in Methods. Lane1, PW2; lane 2, PW2 X S17.1 (pLAFR3); lanes 3-8, PW2 X S17.1(pRK415); lane 9, CsCl -purified pRK415.

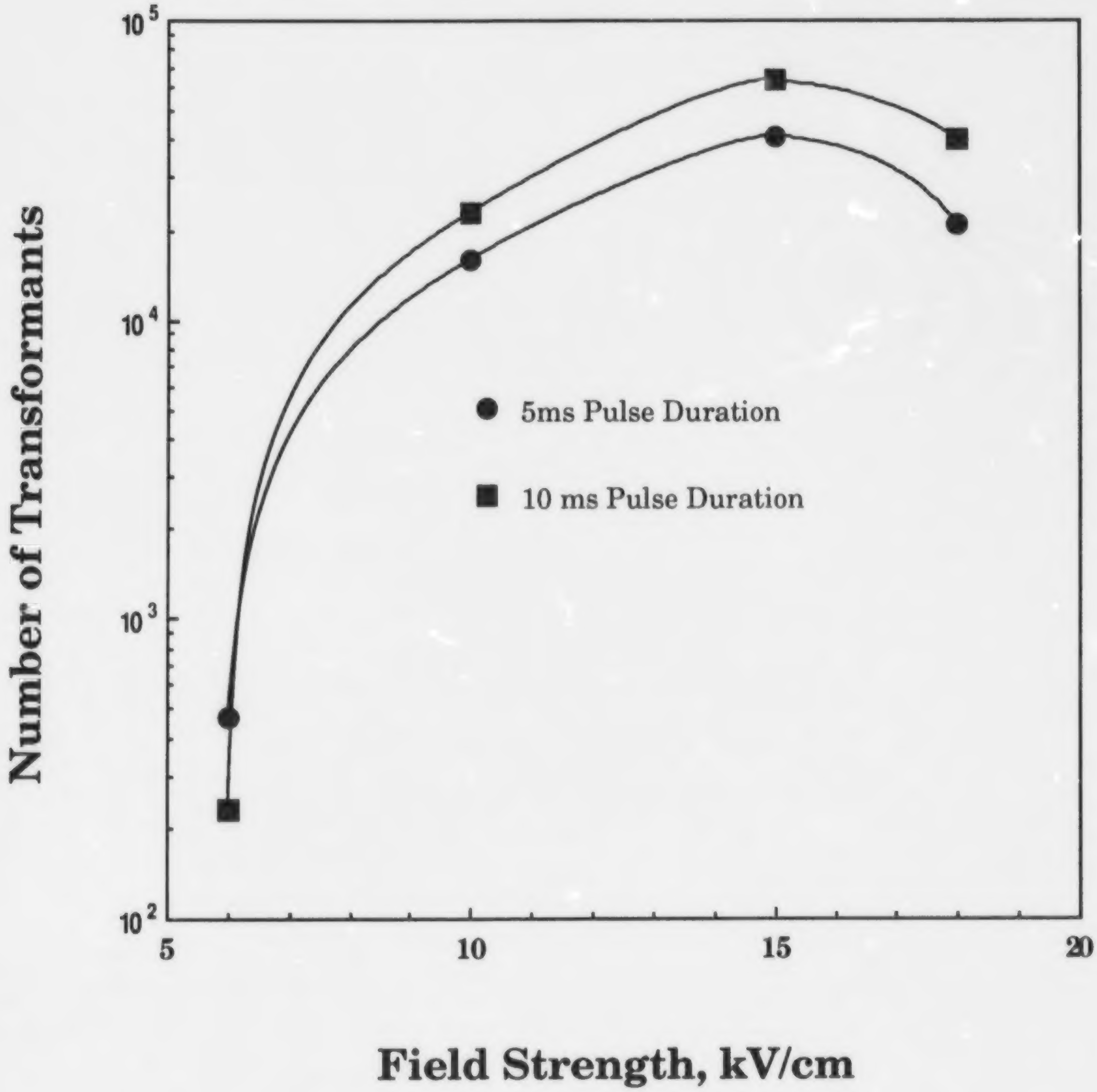

Fig. 2 Effect of increasing field strength on electroporation of PW1.

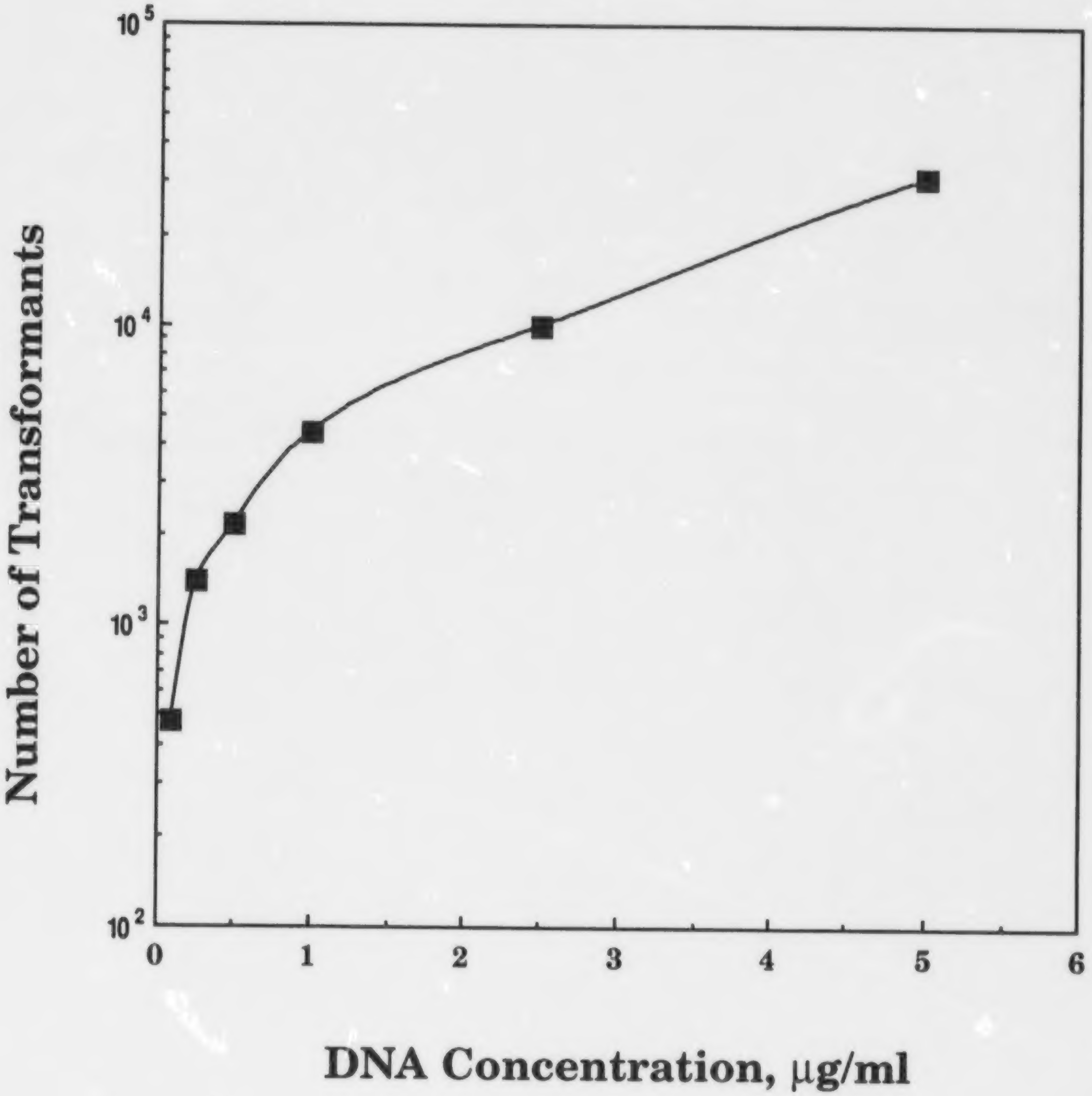

Fig. 3 Effect of DNA concentration on electroporation of PW1.

CHARACTERISATION OF THE MOBILIZATION REGION OF A *THIOBACILLUS FERROOXIDANS* PLASMID

Peter C.K. Lau, Paul Zanga and Marc Drolet*

Genetic Engineering Section, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Ave, Montreal, Quebec, Canada, H4P 2R2

ABSTRACT

Mobilization is a form of conjugation in which a plasmid (nonconjugative) that is deficient in transfer genes is transferred with, or assisted by a conjugative plasmid. The components for the mobilization function of a plasmid DNA include a *trans*-acting sequence coding for mobilization proteins and a cis-acting sequence denoted the origin of transfer or *ori*T. In this study we report the identification of a DNA region of the *Thiobacillus ferrooxidans* plasmid pTF1 required for conjugative mobilization in the presence of the helper plasmid R751. As a result of genetic analysis and DNA sequencing, two of the predicted open reading frames which would encode proteins of calculated molecular mass, 11.4 kilodalton (kDa) and 42.6 kDa, were found to be involved in mobilization. Their amino acid sequences are substantially similar to the previously characterised mobilization proteins of the *Escherichia coli* IncQ plasmid RSF1010. Sequence conservation between plasmids pTF1 and RSF1010 include the *ori*T region, a specific site on the plasmid where a nick in a DNA strand occurs, prior to transfer of DNA from donor to recipient bacteria. The identification of two mobilization (*mob*) genes in pTF1 represents the first report of gene characterisation that are of plasmid origin from the genus *Thiobacillus*.

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* Corresponding author
FAX (514)-496-6232
Tel (514)-496-6325

CARACTÉRISATION DE LA RÉGION DE MOBILISATION D'UN PLASMIDE DE *THIOBACILLUS FERROOXIDANS*

*Peter C.K. Lav**, *Paul Zangu et Marc Drolet*

Section de Génie Génétique, Institut de Recherche en Biotechnologie
Conseil National de Recherche du Canada, 6100 avenue Mont-Royal,
Montréal (Québec) H4P 2R2 Canada

RÉSUMÉ

La mobilisation est une forme de conjugaison dans laquelle un plasmide qui n'a pas les gènes de transfert est transféré en même temps ou avec l'aide d'un plasmide de conjugaison. La mobilisation d'ADN plasmidique requiert une séquence codant pour les protéines de mobilisation en trans et une séquence dénommée l'origine de transfert ou oriT qui agit en cis. Dans cette étude, nous rapportons l'identification d'une région d'ADN dans le plasmide pTF1 de *Thiobacillus ferrooxidans* nécessaire à la mobilisation de gènes par conjugaison en présence du plasmide R751. Suite aux analyses génétiques et au séquençage de l'ADN, deux des cadres de lecture ouverts codant pour des protéines de masse moléculaire de 11.4 kilodalton (kDa) et de 42.6 kDa, se sont avérés être impliqués dans la mobilisation. Leurs séquences en acides aminés sont semblables aux protéines de mobilisation du plasmide RSF1010 de *Escherichia coli* IncQ caractérisées préalablement. Les séquences conservées chez les plasmides pTF1 et RSF1010 comprennent la région oriT, un site spécifique sur le plasmide ou se produit une coupure dans un brin d'ADN juste avant le transfert de l'ADN des bactéries donatrices aux bactéries réceptrices. L'identification de deux gènes de mobilisation (mob) chez pTF1 représente la première communication concernant la caractérisation de gènes d'origine plasmidique chez *Thiobacillus*.

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* Auteur à qui toute correspondance doit être adressée
FAC (514) 496-6232
TÉL (514) 496-6325

INTRODUCTION

Plasmids are extrachromosomally replicating molecules of DNA which are almost ubiquitously found among prokaryotes. The genes which they carry confer a variety of phenotypes on their bacterial hosts. These phenotypes include resistance to antibiotics or metal ions, toxin production and determinants pertaining to replication and bacterial conjugation. The small size of plasmids in general and their maintenance in multiple copies per bacterial chromosome have facilitated their utility as cloning vectors; they are the major vehicles for the transfer of genes between bacteria.

Numerous strains of *Thiobacillus ferrooxidans* have been found to contain plasmids (Martin *et al.*, 1981; Rawlings *et al*, 1983; Holmes *et al*, 1984; Sanchez *et al*, 1986). The most characterised *T. ferrooxidans* plasmid is that of the 12.4 kilobase-pair (kb) pTF-FC2 isolated from the *T. ferrooxidans* strain FC2. Using this plasmid, it was first demonstrated the functional expression of a *T. ferrooxidans* origin of replication (*ori*V) in the heterotroph *Escherichia coli* (Rawlings *et al*, 1984). Subsequently, the ability of pTF-FC2, as well as two other plasmids, to be mobilized among *E. coli* strains was also shown (Rawlings and Woods, 1985). Because these plasmids are intrinsically nonconjugative, mobilization was effected by the broad host range IncPαplasmid RP4 which provides the transfer (tra^+) function. The IncN plasmid R46 was also able to mobilize *T. ferrooxidans* plasmids among *E. coli*, but at a lower frequency than RP4. For the plasmid pTF-FC2, the mobilization region was located on a 5.3 kb DNA fragment. In this study, the identification of two divergently transcribed *mob* genes encoded in the pTF1 plasmid of *T. ferrooxidans* ATCC 33020 is presented. An analysis of the origin of transfer (*ori*T) site situated in the intergenic space of the two *mob* genes is also presented. Furthermore, a sequence relationship of pTF1 with the broad host range IncQ plasmid RSF1010 is established.

Materials and Methods

Bacterial strains and plasmids. The *E. coli* strains were HB101 (*rec*A13, Sm^r; Maniatis *et al*, 1982), TG45 (HB101, Rif^r; gift of G. Tardif) and DH5 (*rec*A1; Hanahan, 1985). Plasmids used were pTF100 (*Hind*III-cleaved pTF1 DNA inserted in pBR322, Ap^r, Mob^+; Holmes *et al*, 1984), R751 (IncP1, tra^+, Tp^r, gift of R. Meyer), pBR328 (Ap^r, Tc^r, Cm^r, Soberon *et al*, 1980). Derivatives of pTF1 DNA in pBR328 are outlined in Fig. 1.

Genetic and molecular techniques. General recombinant DNA techniques are according to Maniatis *et al.*, (1982). Conjugation experiments were carried out essentially as described by Rawlings and Woods (1985).

Mapping of the relaxation nick site. The procedure of Guiney and Helinski (1979) in the preparation of plasmid-protein relaxation complex (relaxosome) was followed but scaled up to 250 ml cell culture. The plasmid nick site was mapped by linearizing the relaxed plasmid with various restriction endonucleases and separating the single-stranded DNAs on an 0.8% alkaline agarose gel (Pansegrau *et al.*, 1988). For electropheresis, the buffer contains 60 mM potassium triphosphate, pH 12.0 and 0.5 mM EDTA (A. Vaisius, unpublished data). Following electrophoresis, the gel was stained with ethidium bromide in 90 mM potassium triphosphate pH 6.0 and then photographed under UV in the usual manner.

Results and Discussion

Plasmid deletion and genetic analysis

The *T. ferrooxidans* strain ATCC 33020, grown in the presence of uranium, was previously found to harbor 3 plasmids; the smallest of these is pTF1 which is 6.7 kb in size, and like others, it has no readily identifiable phenotype (Holmes *et al*, 1984). Rawlings and Woods (1985) have cloned a similar, if not identical, plasmid from *T. ferrooxidans* ATCC 33020 which they designated pTF33020-1. When cloned in pBR325, pTF33020-1 was found to be efficiently mobilized by the broad host range IncPα group plasmid RP4 and less efficiently by the IncN plasmid R46. A similar observation was obtained with the 12.4 kb pTF-FC2 plasmid from *T. ferrooxidans* strain FC-2, in which case the mobilization region was located on a 5.3 kb plasmid fragment. In order to localize and identify a region of plasmid pTF1 involved in the conjugative mobilization process, several deletion derivatives of pTF1 (Fig. 1) were constructed in the *mob*-deficient pBR328 (Soberon *et al.*, 1980). The IncPβ plasmid R751 was used as the 'helper' plasmid. The donor *E. coli* strain was HB101 and the recipient strain was TG45, a rifampicin-resistant derivative of HB101. Conjugation conditions were carried out essentially as described by Rawlings and Woods (1985). The transfer frequency is calculated as the number of trimethoprim-resistant (200 μg/ml; R751) transconjugants per donor cell. Mobilization frequency is calculated as the number of transconjugants (pTF1 derivatives in pBR328) per donor cell. The mobilization ratio is the mobilization frequency divided by the transfer frequency. The IncPβ plasmid R751 (like the IncPα RP4, not shown) was found to mobilize the transfer of several derivatives of pTF1 in pBR328 at high frequencies (Fig. 1, those marked as mob$^+$). The mobilization ratio is in the range of 3.4-4.5 x 10^{-2} to 5.2×10^{-2} , the latter value is that obtained with the entire pTF1 DNA cloned in pBR328. On average, the transfer frequency of R751 is 0.1 per donor cell. In Fig. 1, those derivatives marked as mob$^-$ indicate a mobilization ratio $< 10^{-7}$. Of the deletion plasmids obtained and analyzed, the smallest region of the pTF1 plasmid that is required for mobilization is the fragment spanning *Xho*II (at coordinates 3.868 proximal to the *Bgl*II site) to the *Hin*dIII site (coordinate 6.657; arbitrary end or start of plasmid numbering). The DNA sequence of the 2797 base-pair (bp) *Xho*II-*Hin*dIII fragment has been determined and will be described elsewhere. Some features of the mobilization region are described below.

The mobilization genes of pTF1

Six open reading frames (ORF's 2 — 7) with the potential of coding for proteins, ranging in size from 75 amino acids (ORF3) to 378 amino acids (ORF 2), were predicted from DNA sequence analysis of the 2.8 kb *Xho*II-*Hin*dIII fragment (Fig.1). Except for ORF7 which has a predicted isoelectric point of 5.5, all other ORF's are presumably to be basic proteins. When the amino acid sequences of ORF's 2 — 7 were searched for similarity against the protein sequences in the various databases, ORF2 and ORF6 were found to have a significant degree of sequence homology with two of the three known mobilization proteins, viz. MobA and MobC, of the *E. coli* plasmid RSF1010 (Fig 2). Between ORF6 (98 amino acids) and MobC (94 amino acids), an overall 52.7% sequence similarity was observed; most of the sequence conservation is confined to the C-terminal portions of the proteins (not shown). A comparison of ORF2 with the RSF1010 MobA protein (709 amino acids) shows that there is 48.7% sequence similarity. However, the conserved region in MobA is confined to the N-terminal protein portion which is active in plasmid mobilization. The C-terminal 323 amino acid domain of MobA has a separate primase activity (Scholz *et al*, 1989).

By virtue of sequence homology as described above and taken together with the deletion analysis in Fig. 1, it is clear that there are at least two mobilization genes (ORF2 and ORF6) in pTF1. The involvement of ORF2 and ORF6 in mobilization is established by deletions at *Eco*47 III (base no. 5594) and *Xho*II (base no. 4622 proximal to *Sau*3A) which interrupt ORF2 and ORF6 respectively resulting in a *mob*-deficient phenotype (Fig. 1). It is not clear, however, about the possible involvement of ORF's 3, 4 and 5 in pTF1 mobilization, largely because of the overlapping nature of these putative proteins with ORF2. None of these ORF's has sequence similarity with MobB (Fig.2) or other overlapping ORF's within the mobilization region of RSF1010 (Derbyshire *et al*, 1987).

The pTF1 *ori*T site

The sequence relatedness of the mobilization systems of plasmids RSF1010 and pTF1 is extended to the intergenic space separating the two divergently transcribed genes. The intergenic region of ORF2 and ORF6 of pTF1 is only one nucleotide shorter than that of *mob*A and *mob*C of RSF1010 (Fig. 2). It has been established previously that in RSF1010 and its presumed identical R1162 plasmid system

(Barth and Grinter, 1974), a region referred to as the origin of transfer or *ori*T is located in the intergenic space. Specifically, this is a DNA region where a strand-specific nick or break occurs leading to transfer of DNA from donor to recipient cell during conjugation. Genetically, the *ori*T is defined as the region required in cis to the DNA that is to be transferred (Willetts and Wilkins, 1984). Brasch and Meyer (1987) localized the *ori*T site of R1162 (RSF1010) to a 38 bp sequence. Subsequently, Meyer (1989) showed that the *Hin*PI recognition sequence within the 38 bp fragment is the transfer-dependent cleavage or nick site. This site is adjacent to an inverted repeat sequence of 10 bp with one mismatch. Fig. 3 shows that within the intergenic space of pTF1 ORF2 and ORF6, there are sequences identical to those of RSF1010 in the minimal 38 bp *ori*T region. Moreover, a common 10 bp inverted repeat sequence with one mismatch can be drawn. Presumably, the inverted repeat sequence can serve as a specificity determinant for the interaction with the mobilization protein(s). This is the case in plasmid RP4 in which the plasmid-coded TraJ protein (13.3 kDa) is found to bind to a 19 bp inverted repeat sequence within the *ori*T site (Ziegelin *et al*, 1989).

It is necessary to demonstrate the location of the pTF1 nick site. To do this, a supercoiled DNA-protein relaxation complex or relaxosome of pTF1 4 is isolated by the gentle cell lysis technique (Guiney and Helinski, 1979). The pTF14 plasmid is 7.705 kb in size and contains the 3.15 kb *Bgl*II-*Hin*dIII pTF1 DN A in pBR328. The supercoiled DNA is relaxed by treatment with sodium dodecyl sulfate (SDS) and proteolytically digested with pronase to convert to the open circular form (Fig. 4; for review see Willetts and Wilkins, 1984). To analyze the nick site, the gel electrophoretic assay developed by Pansegrau *et al*., (1988) was followed. The relaxed pTF14 DNA was linearized with either *Stu*I, *Hind*III, *Eco*RI or *Pst*I (Fig. 5). In each case the single-stranded DNA species are analyzed in an alkaline agarose gel. The presence of three distinct bands on the gel (a common full length linear and two smaller fragments, the sum of which equals that of the full length linear) demonstrates that the nick must have occurred at a unique site. Accordingly, calculation of fragment sizes located the nick site of pTF1 DNA within the intergenic sequence of ORF2 and ORF6 around nucleotide position 4860 in the region of *ori*T (Fig. 2). To more accurately localize the nick site and to determine the DNA strand specificity, DNA sequencing has also been carried out (not shown). The specific nick was found in the middle of the *Hin*PI recognition sequence (Fig. 4) at nucleotide position 4898 of the pTF1 sequence. This places the nick 118 bases from the initiation codon of ORF6 and 79 bases from the initiation codon of ORF2 (in the complementary strand).

Conclusion

Short of a direct transformation system, using naked plasmid DNA, for the introduction of potentially useful genetic traits into *T. ferrooxidans* , the discovery of *T. ferrooxidans* recombinant plasmids mobilizable by the broad host range plasmids such as RP4 or R751 is a relievable alternative (Rawlings and Woods, 1985; this study). The recent demonstrations of plasmid transfer from *Agrobacterium* into plant cells by the *mob* function of RSF1010 (Buchanan-Wollaston *et al*., 1987) and the bacterial-yeast conjugation mediated by the ColE1 *mob* function (Heinemann and Sprague, 1989) underscore the importance of the mobilization function of a bacterial plasmid system in promoting gene exchange.

To our knowledge, the identification of two mobilization genes of the pTF1 plasmid represents the first report of plasmid gene characterisation from the genus *Thiobacillus*. The relatedness of the pTF1 *mob* genes to those of the *E. coli* plasmid RSF1010 (Guerry *et al*, 1974) or *Pseudomonas aeruginosa* plasmid R1162 (Barth and Grinter, 1974) suggests a common ancester. Also, considering the sequence conservation of several *T. ferrooxidans* chromosomal genes with those of other bacteria (review: Woods *et al*, 1986) *T. ferrooxidans* may be viewed as an ordinary soil bacteria trapped in an acidic environment, a niche in which the organism lives to adopt. It is important to learn and understand more of the basic biology or genetics of the economically relevant *T. ferrooxidans* in order to benefit from a possible enhanced leaching capabilities of these microorganisms via genetic manipulations (Woods *et al*., 1986; Hutchins *et al*., 1986).

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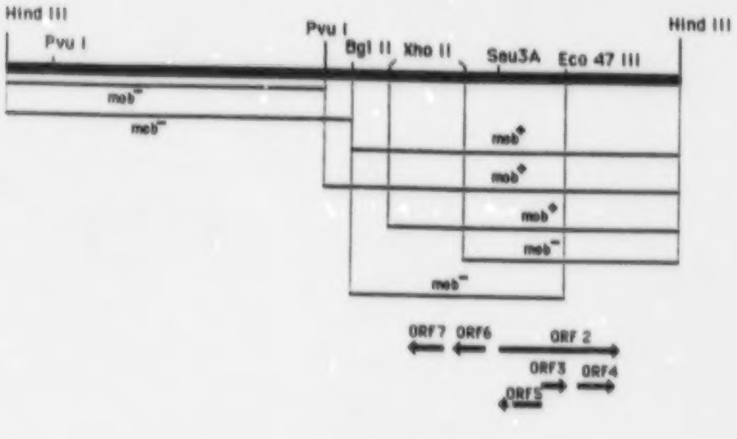
Hind III
Pvu I
Pvu I
Bgl II
Xho II
Sau3A
Eco 47 III
Hind III
mob⁻
mob⁻
mob⁺
mob⁺
mob⁺
mob⁻
mob⁻
ORF7
ORF6
ORF 2
ORF3
ORF4
ORF5

Fig. 1 Deletion analysis of pTF1 plasmid for mobilization function.

The restriction map of pTF1 is according to Holmes et al (1984) and this study. The directions of transcription of the predicted open reading frames (ORF) within the *mob*-positive region are as indicated.

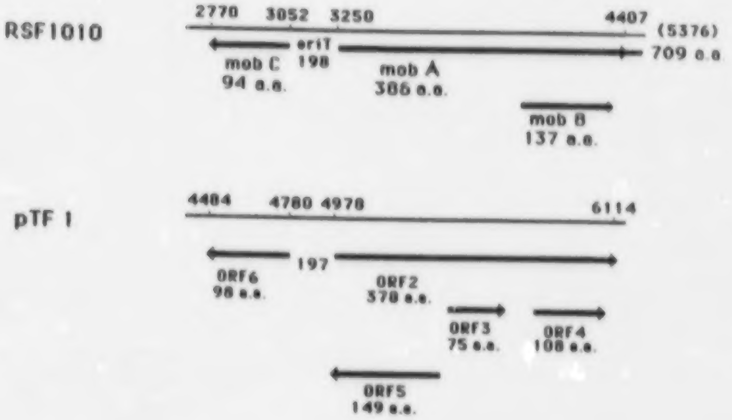
RSF1010
2770
3052
3250
4407
(5376)
oriT
198
mob C
94 a.a.
mob A
386 a.a.
709 a.a
mob B
137 a.a.
pTF 1
4484
4780
4978
6114
197
ORF6
98 a.a.
ORF2
378 a.a.
ORF3
75 a.a.
ORF4
108 a.a.
ORF5
149 a.a.

Fig. 2 Comparison of gene arrangements in the mobilization regions of plasmids RSF1010 and pTF1.

The size of the protein in amino acids (a.a) each ORF could encode is indicated. The full length of MobA is 709 a.a. (Scholz *et al*, 1989). The map locations are according to the complete sequence of RSF1010 (Scholz *et al.*, 1989) and pTF1 (this laboratory, unpublished). In RSF1010, other overlapping ORF's within the *mob* region (besides those shown above) exist (Derbyshire *et al.*, 1987).

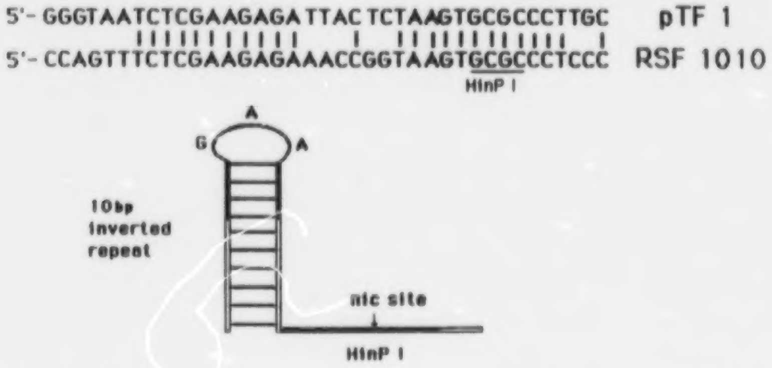
5'- GGGTAATCTCGAAGAGATTAC TCTAAGTGCGCCCTTGC
pTF 1
5'- CCAGTTTCTCGAAGAGAAACCGGTAAGTGCGCCCTCCC
RSF 1010
HinP I
A
G
A
10bp inverted repeat
nic site
HinP I

Fig. 3 Sequence alignment of the minimal 38 bp *ori*T region of RSF1010 with pTF1 DNA.

Identical nucleotides are paired. Both sequences can be folded into a common hairpin structure as shown. In the schematic representation, the dark lines indicate regions of identical sequences. The sequence of RSF1010 is according to Brasch and Meyer (1987).

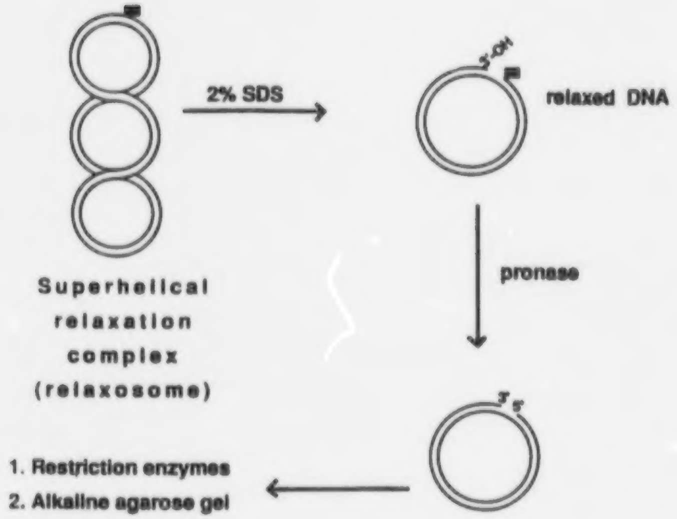
2% SDS
3'-OH
relaxed DNA
pronase
Superhelical relaxation complex (relaxosome)
3'
5'
1. Restriction enzymes
2. Alkaline agarose gel

Fig. 4 Steps towards the preparation of relaxed plasmid DNA for assay of site-specific nick.

The filled rectangle represents one or more relaxation proteins associated with the superhelical DNA; in the open circular form, the protein(s) is covalently linked to the 5'-end of the nicked strand (Guiney and Helinski, 1979).

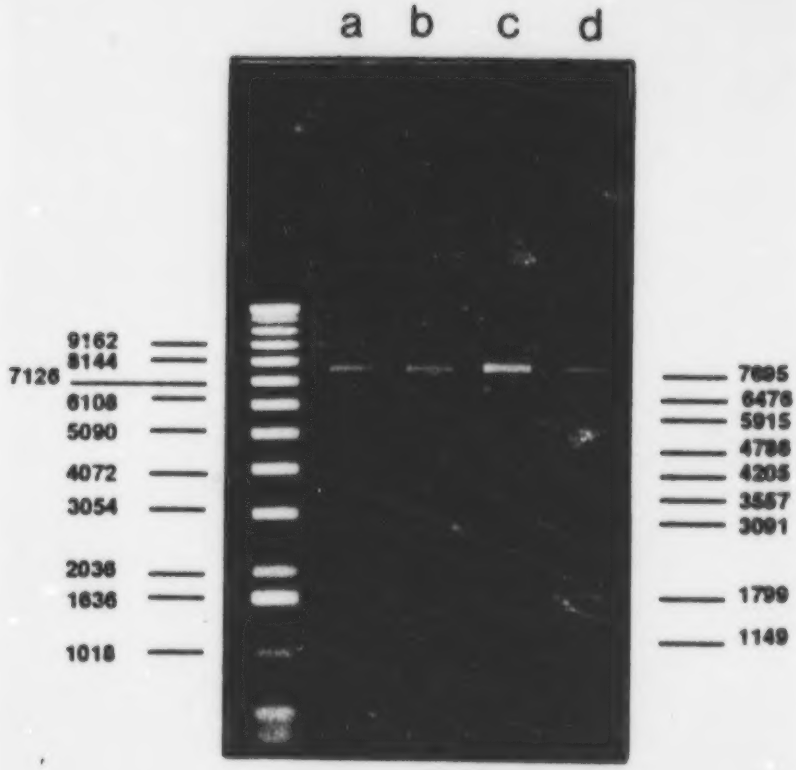
a
b
c
d
9162
8144
7126
6108
5090
4072
3054
2036
1636
1018
7695
6476
5915
4788
4205
3557
3091
1799
1149

Fig. 5 Alkaline agarose gel electrophoresis of single-stranded DNA species generated by a site-specific relaxation of pTF14 DNA and linearisation by restriction enzymes a) *Stu*I, b) *Hind*III, c) *Eco*RI and d) *Pst*I. The left lane indicates a DNA size standard.

STUDIES ON A BACTERIOPHAGE WHICH INFECTS MEMBERS OF THE GENUS *ACIDIPHILIUM*

T. E. WARD, M. L. ROWLAND, D. F. BRUHN,
C. S. WATKINS and F. F. ROBERTO

Biotechnology
Idaho National Engineering Laboratory Idaho Falls, ID 83415-2203

ABSTRACT

The genus *Acidiphilium* contains acidophilic, heterotrophic, aerobic, gram-negative eubacteria, which are most commonly isolated from acidic mine drainage environments. A bacteriophage, termed øAc1, infecting some strains of the genus *Acidiphilium* has been discovered, and several of its characteristics have been investigated. The interaction of øAc1 with host bacteria was investigated using one-step growth experiments and single burst experiments. One step growth experiments show that the latent period of this phage is approximately three hours under our standard conditions. A burst size of approximately 62 phage per cell was calculated from the results of a single burst experiment. The nucleic acid of øAc1 is double-stranded DNA, approximately 97,000 base pairs in length. Restriction enzyme analysis suggests that the virion DNA may be modified. Several results indicate that øAc1 is a temperate phage. The plaques appear to be turbid, and cells which are resistant to further phage infection can be isolated from the plaques. These cells contain the phage and behave like the original donor cells. In addition, Southern blot analysis shows that øAc1 prophage DNA is integrated into the bacterial genome during lysogenic growth. An understanding of phage øAc1 and its use as a genetic tool will increase our understanding of the genetics of the genus *Acidiphilium*.

ÉTUDES SUR UN BACTÉRIOPHAGE QUI INFECTE LES MEMBRES DU GENRE *ACIDIPHILIUM*

T.E. Ward, M.L. Rowland, D.F. Bruhn, C.S. Watkins et F.F. Roberto

Biotechnologie
Laboratoire National de Génie de l'Idaho
Idaho Falls, ID 83415-2203 É.-U.

RÉSUMÉ

Le genre *Acidiphilium* est constitué d'eubactéries gram-négatives acidophiliques, hétérotrophes et aérobies, qui sont les plus souvent isolées à partir d'environnements miniers acides. Un bactériophage désigné Acl, capable d'infecter certaines souches du genre *Acidiphilium* a été découvert, et plusieurs de ses caractéristiques ont été étudiées. L'intéraction du plasmide Ac1 avec des bactéries hôtes a été étudiée à l'aide d'expériences avec croissance de cultures synchrones et d'expériences avec une seule période d'éclatement. La croissance de cultures synchrones montrent que la période de latence de ce phage est approximativement de trois heures sous nos conditions standards. Une quantité approximative de 62 phages par cellules a été calculée à partir des résultats d'une expérience avec un seul éclatement. L'acide nucléique d'Acl est composé d'ADN à double-brins, de longueur approximative de 97,000 paires de bases. L'analyse à l'aide d'enzymes de restriction suggère que l'ADN du virion peut-être modifié. Plusieurs résultats indiquent qu'Acl est un phage tempéré. Les plaques sont turbides et les cellules qui sont résistantes à une infection phagique ultérieure peuvent y être isolées. Les cellules contiennent le phage et se comportent comme les cellules donnatrice originales. En plus, l'analyse de Southern montre que l'ADN du prophage Acl est intégré dans le génome bactérien durant la croissance lysogène. Une connaissance du phage Acl et son utilisation comme outil génétique va nous permettre d'augmenter notre compréhension sur la génétique du genre *Acidiphilium*.

INTRODUCTION

Acidophilic bacteria are intimately involved in leaching of metals from ores and in production of acid mine drainage. They may also find application in the removal of pyritic sulfur from coal. The most intensively studied such bacterial species has been *Thiobacillus ferrooxidans*. However, other acidophilic bacteria are present in these environments. Their contributions to these various processes are not well understood. These bacteria include members of the genus *Acidiphilium* – acidophilic, aerobic, heterotrophic, gram-negative eubacteria. There is evidence suggesting that these acidophilic heterotrophs can increase the rate of *T. ferrooxidans*' attack on inorganic sulfides, which is involved in all three processes noted above.

The genetic characteristics of these acidophilic bacteria have not been extensively investigated. As part of our study of the genetic properties of the genus *Acidiphilium*, a search was made for an endogenous bacteriophage. Such a bacteriophage was discovered by performing a series of pairwise crosses with different *Acidiphilium* strains (Ward *et al.*, 1989a, b). We have termed this phage øAc1. This report describes several characteristics of øAc1 and its interaction with its host cells.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were reagent grade and most were purchased from Fisher Scientific. The yeast extract was from BBL and Difco, and the agarose was from Sigma. Restriction enzymes were from Boehringer, N. E. Biolabs and Promega, and digestions with these enzymes were performed according to manufacturers recommendations. Deionized water from a Barnstead "Nanopure" unit was used to prepare all media.

Bacterial Strains

Acidiphilium strains CM1, CM3, CM3A, CM4, CM4A, CM5, CM7, CM9 and CM9A were isolated from water collected at the Blackbird cobalt mine, southwest of Salmon, Idaho (Thompson, D. L. and Wichlacz, P. L., unpub.).

The other *Acidiphilium* strains used in this study were isolated from acidic mine drainage sites in central Pennsylvania and were supplied by P. L. Wichlacz (Wichlacz and Unz, 1981; Wichlacz *et al.*, 1986). Strains KLB, LHet, OP and PW2 are ATCC strains 35903, 33463, 35905 and 35904, respectively.

Media, Preparation of Phage Stocks and Plaque Assays for Phage

Media used, preparation of plate stocks of phage, and the plaque assay used to titer phage have been previously described (Ward, *et al.*, 1989a, b). In experiments using antibiotics, and some more recent experiments, the growth medium was modified by leaving out aluminum and manganese, reducing the $MgSO_4$ to 2 mM and the $CaCl_2$ to 0.1 mM, adding 2.6 mM $FeSO_4$ and raising the pH to 3.5 (MAS medium). Attachment buffer contained 1 mM $(NH4)_2SO_4$, 2 mM KCl, 10 mM $MgSO_4$, 10 mM $CaCl_2$, 0.1% glycerol and 0.01% yeast extract, pH 5.0.

One Step Growth Experiment

$3X10^7$ pfu of phage were mixed with $4X10^7$ cells (washed and resuspended in 1 mM $(NH_4)_2SO_4$, 2 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.7 mM $MnSO_4$, 4 mM KOAc, pH 5.0) in a volume of 2.3 mls, and attachment was allowed to proceed for 20 minutes. 100 μl of the mixture was diluted into 99.9 mls of glycerol salts medium (Ward *et al.*, 1989a, b) without aluminum and yeast extract and shaken at 32°C for several hours. At various times, one ml samples were removed, filtered through 0.45 μm filters to remove cells, and assayed to determine the titer of free phage.

Single Burst Experiment

$6X10^7$ pfu of phage were added to $1.4X10^8$ cells, (washed and resuspended in attachment buffer), in a total volume of 4.0 mls, and attachment was allowed to proceed for 20 minutes. The infected cells were washed two times in attachment buffer, diluted 10^{-3} fold into MAS medium, and 10 mls were incubated at 32°C for 100 minutes. The mixture was then diluted ten fold to approximately $3.5X10^3$ cells/ml (approximately five lytically infected cells/ml), and about one hundred aliquots (100 μl) were distributed to 13 X 100 mm glass tubes. These tubes were shaken for an additional 120 min. at 32°C to allow lysis, and each aliquot was then titered to determine the number of phage present.

Isolation and Analysis of Phage Nucleic Acid

Phage stocks ($2\text{-}3x10^9$ pfu) were pelleted in a Beckman SW40 rotor at 20,000 rpm and 4°C for 60 minutes. The supernatant was decanted and the phage pellet was gently resuspended in 50mM Tris, 50mM EDTA pH 8.0. The solution was extracted with one volume of phenol (equilibrated with Tris pH 8.0) and one volume of chloroform. The phases were separated by centrifugation, and the extraction was repeated twice more. The DNA was dialyzed extensively against TE (10mM Tris, 1mM EDTA pH 7.5). Restriction enzyme and S1 nuclease digestions were carried out according to the manufacturers instructions. Field-inversion gel electrophoresis was performed as previously described (Ward, et al, 1989b; Carle et al, 1986).

Southern Blot Analysis

DNA samples were electrophoresed on a 0.8% agarose gel in 0.5X TBE at 7V/cm for 14 hours using Program 3 of the PPI 200 (a reverse incremental cycle of 0.05-1.6 seconds and a forward incremental cycle of 0.15-4.8 seconds). After EtBr staining and photography, the gel was soaked in 0.25M HCl for 10 minutes and then transferred to a nitrocellulose membrane as described in Maniatis et al (1982). The filter was rinsed for 5 minutes in 5X SSC (0.75M NaCl, 0.075M sodium citrate) and baked for 2 hours at 80°C. The filter was then hybridized to øAc1 DNA, which had been labeled with ^{32}P by nick translation (Maniatis et al, 1982), followed by washing and autoradiography.

RESULTS

A temperate or lysogenic phage, termed øAc1, has been found in *Acidiphilium* cells by performing a series of pairwise crosses (Ward *et al.*, 1989a, b). As expected, some combinations of strains resulted in formation of plaques and several did not. Strains CM1, CM3A and CM9A proved to be donors (lysogens) and strains CM3, CM5, CM9 and PW2 proved to be sensitive to the phage (Ward et al, 1989a, b). The following experiments were performed to answer questions that typically arise concerning a newly discovered phage, such as: What is the length of the life cycle and the average burst size? What type of nucleic acid does the phage possess, and what is its size? What is the nature of the phage's interaction with the cell?

Determination of the øAc1 Latent Period (One Step Growth Experiment)

A one step growth experiment (Luria and Darnell, 1967) was performed in order to determine the length of the phage life cycle (the latent period). Cells were infected and samples were taken at various times and assayed to determine the number of free phage present. The results (Figure 1) show that the titer of free phage dropped gradually over the course of 2.5 hours, indicating that the adsorption process is relatively slow. The results also show a rise in titer at about three hours, which indicates that this is the length of the phage life cycle under these conditions of medium, growth temperature, host cells, etc.

Determination of the øAc1 Burst Size (Single Burst Experiment)

A single burst experiment was performed to determine the average number of phage released from a lysing bacterium (the burst size). In this experiment, cells were infected and incubated for part of the life cycle, after which they were diluted and divided into a large number of aliquots, each of which contained, on the average, slightly less than one lytically infected cell. The aliquots were then incubated until the cells lysed, after which all aliquots were assayed for released phage. The results (Figure 2) show a wide variation from sample to sample in the number of phage released, as expected (Luria and Darnell, 1967). Analysis of the data (Luria and Darnell, 1967) shows that the average burst size is approximately 62 phage/cell.

Characterization of øAc1 Nucleic Acid

Several experiments were performed to investigate the nature of øAc1 virion nucleic acid. The nucleic acid is completely digested by DNAse I, but not by RNAse A (data not shown). Unheated øAc1 nucleic acid is not digested by S1 nuclease, but heat treated øAc1 nucleic acid is completely digested by S1 (data not shown). Also, øAc1 virion nucleic acid can be digested by restriction endonucleases EcoRV, SmaI, SphI, Sau3AI, and RsaI (Table 1). These experiments lead to the conclusion that the nucleic acid of øAc1 is double-stranded DNA.

The molecular weight of øAc1 DNA was estimated using field-inversion gel electrophoresis (Carle *et al.*, 1986). The DNA's of bacteriophages λ, P1 and T5 were used as molecular weight standards. A molecular weight of 97,000 base pairs was calculated from the known molecular weights of P1 and T5 DNA (Figure 3).

Indirect Evidence for Modification of øAc1 DNA

øAc1 DNA was treated with a series of restriction enzymes in an attempt to produce a restriction map. Several restriction enzymes (Table 1) failed to cut the virion DNA when reactions were carried out according to manufacturers specifications. The digestions with HindIII and EcoRI were repeated with the addition of pBR328 DNA to the reaction mixtures. Again the øAc1 DNA was not cut, while the pBR328 DNA was digested, showing that the enzymes were active in these reactions. This indicates either that the DNA is modified in some way and thus protected against enzyme digestion or that there are no recognition sequences for these enzymes in øAc1 DNA. This length of DNA would be expected to contain approximately 22-25 sites for each of these enzymes, which have six-base recognition sequences (except Not I). The chance that this DNA contains no sites for a number of restriction enzymes is thus statistically very remote. This leads to the conclusion that the virion DNA is probably modified in some way.

TABLE 1

RESTRICTION ENZYME DIGESTION OF øAc1 VIRION DNA

RESTRICTION ENZYME	ACTIVE ON PHAGE DNA	RECOGNITION SEQUENCE
Eco RI	−	GAATTC
Hind III	−	AAGCTT
Apa I	−	GGGCCC
Xho I	−	CTCGAG
Not I	−	GCGGCCGC
Sac I	−	GAGCTC
Pvu II	−	CAGCTG
Eco RV	+	GATATC
Sma I	+	CCCGGG
Sph I	+	GCATGC
Rsa I	+	GTAC
Sau 3AI	+	GATC

Evidence that øAc1 is a Temperate Phage

Several lines of evidence suggest that øAc1 is a temperate phage. Cells resistant to the phage can be isolated from plaques produced on sensitive cells by cell-free phage preparations. These resistant cells harbor the phage and behave similarly to the original donor strains (Ward et al, 1989a, b). A Southern blot experiment was performed on undigested virion DNA and chromosomal DNA from sensitive and phage donor cells (lysogens), using labeled phage DNA as a probe. The results (Figure 4) show that the phage DNA sequences in the lysogen run with the bulk of the chromosomal DNA and not where isolated phage DNA runs. This shows that the prophage DNA is integrated into the chromosome during lysogenic growth, and does not exist as a separate, extrachromosomal entity. As expected, there is also no hybridization to the chromosomal DNA of an uninfected, sensitive cell (lane 2, panel "B", Figure 4).

CONCLUSIONS

A bacteriophage which infects some strains of the genus *Acidiphilium* has been discovered and termed øAc1. Some strains of *Acidiphilium* carry the phage and are resistant to superinfection, some strains are sensitive to the phage, and some strains are resistant but do not appear to carry such a phage (Ward et al, 1989a, b). One step growth experiments showed that the latent period of the phage is approximately three hours under our standard growth conditions. The decline in the number of free phage occurs more slowly than with typical bacteriophage, indicating that the process of absorption is relatively slow for this phage. The rise in the number of free phage at the end of the growth cycle, which represents the phage liberated from lysing bacteria, is also much less pronounced than with typical bacteriophage. Presumably this reflects the relatively small number of bacteria that are lytically infected by this phage, estimated from the single burst experiment to be approximately 0.1% of the cells. The average burst size of the phage was calculated to be 62 phage per cell from the single burst experiment.

The nucleic acid of the phage was shown to be double stranded DNA by several criteria. The size of øAc1 DNA was estimated to be 97 kb using field-inversion gel electrophoresis and the DNA's of bacteriophages λ, P1 and T5 as molecular weight standards. This is consistent with earlier results concerning the size of the phage particle. Observations using the electron microscope revealed a head diameter approximately 1.44 times that of lambda, and sucrose gradient centrifugation yielded a sedimentation coefficient approximately 1.57 times that of lambda (Ward et al, 1989a, b).

There is circumstantial evidence that øAc1 DNA is modified. The restriction endonucleases EcoRI and HindIII do not digest øAc1 virion DNA, while pBR328 DNA in the same reaction mixture is digested. This means either that the phage DNA is protected from digestion or that there are no recognition sequences for these enzymes in the DNA. The chance that no such site for either enzyme is present in a 97 kb segment of DNA is statistically very small, thus leading to the conclusion that the phage DNA is probably modified in some way. Recent preliminary results indicate that segments of phage DNA, cloned and amplified in *E. coli*, can be cut by EcoRI (data not shown). This supports the conclusion that the phage DNA is modified.

There is good evidence that øAc1 is a temperate bacteriophage. It was isolated by performing a series of pairwise crosses with different *Acidiphilium* strains, none of which individually gave any indication that they might harbor a bacteriophage. In addition, cells which are resistant to phage infection can be isolated from plaques produced on sensitive cells by cell-free preparations of øAc1. These resistant cells harbor the phage and behave similarly to the original donor strains (Ward et al, 1989a, b). Finally, Southern blot analysis of undigested øAc1 virion DNA and lysogen chromosomal DNA shows that the prophage is present in the high molecular weight chromosomal DNA of the lysogen and thus is integrated into the cellular DNA during lysogenic growth (Figure 4).

Further study of this bacteriophage will provide insights into the genetic mechanisms present in the genus *Acidiphilium*. We hope to be able to modify and use this phage as a genetic engineering vector, much as bacteriophage lambda has been so successfully used with *E. coli*.

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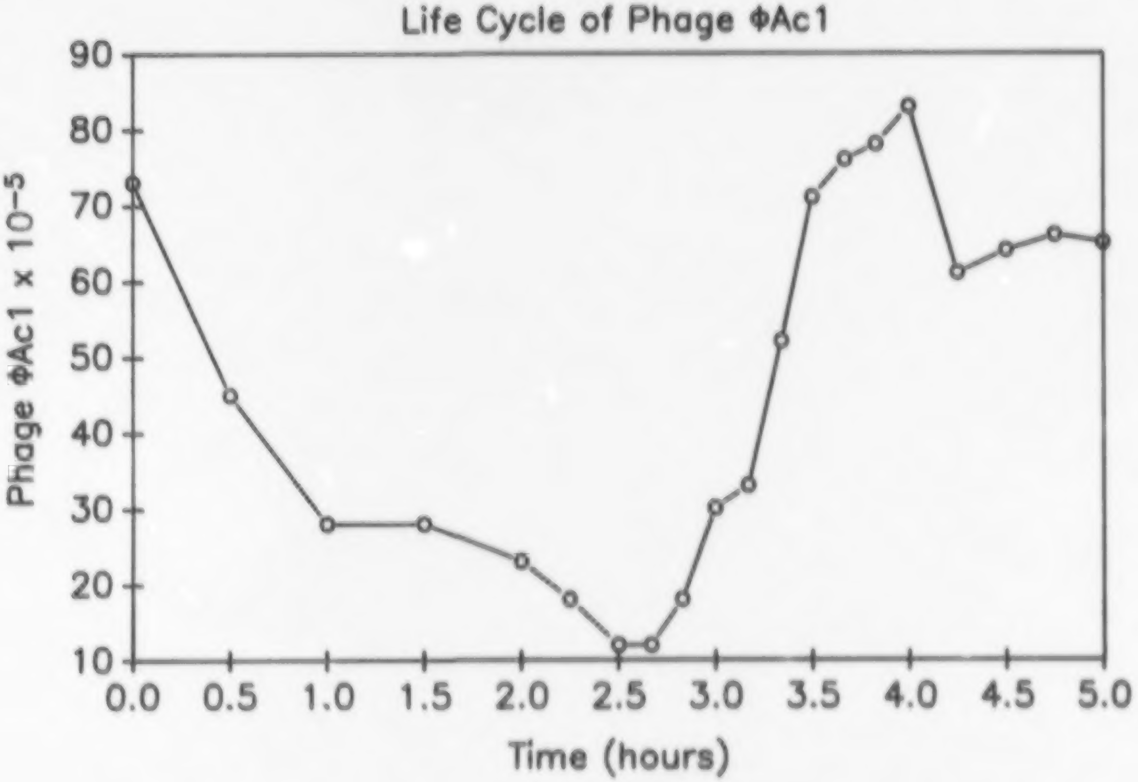

Fig. 1 One Step Growth Experiment. CM9 cells were infected with øAc1 at an MOI of 0.7 phage/cell and shaken in growth medium at 32°C. At various times, one ml samples were removed, filtered to remove cells and assayed to determine the titer of free phage.

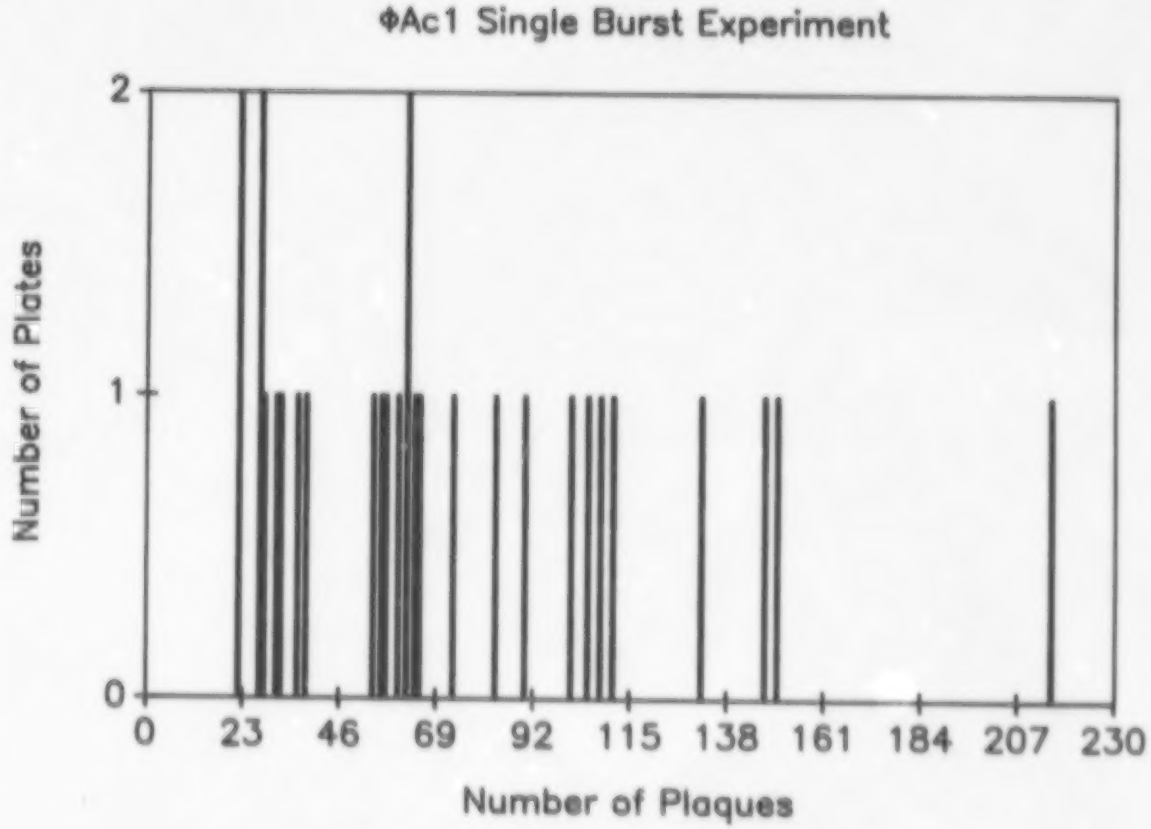

Fig. 2 Single Burst Experiment. CM9 cells were infected with ø Ac1 at an MOI of 0.4 phage/cell and after 120 minutes diluted to approximately 5 lytically infected cells/ml. One hundred aliquots (100 μl) were prepared and shaken until four hours after infection. All aliquots were then titered for phage.

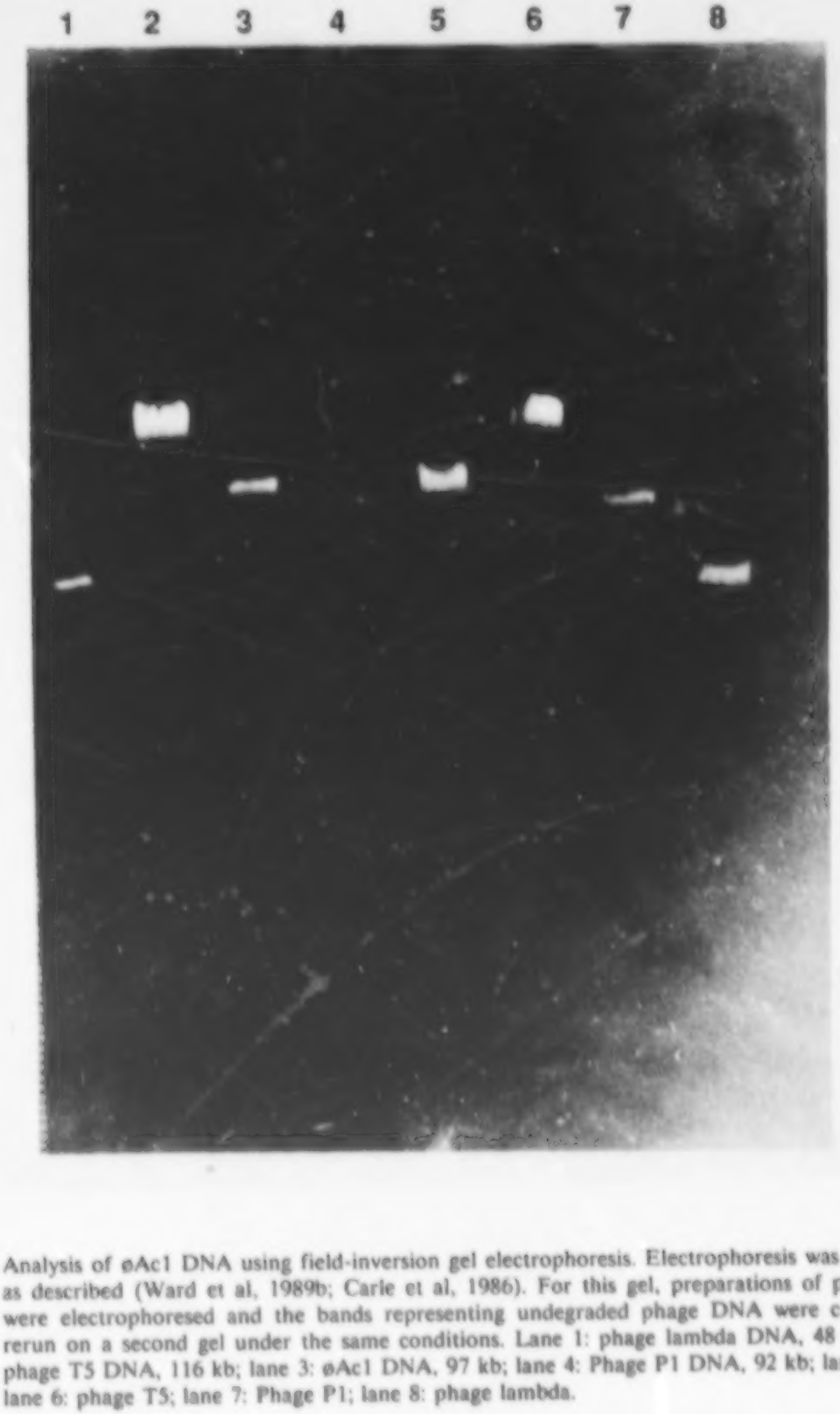

Fig. 3 Analysis of øAc1 DNA using field-inversion gel electrophoresis. Electrophoresis was performed as described (Ward et al, 1989b; Carle et al, 1986). For this gel, preparations of phage DNA were electrophoresed and the bands representing undegraded phage DNA were cut out and rerun on a second gel under the same conditions. Lane 1: phage lambda DNA, 48 kb; lane 2: phage T5 DNA, 116 kb; lane 3: øAc1 DNA, 97 kb; lane 4: Phage P1 DNA, 92 kb; lane 5: øAc1; lane 6: phage T5; lane 7: Phage P1; lane 8: phage lambda.

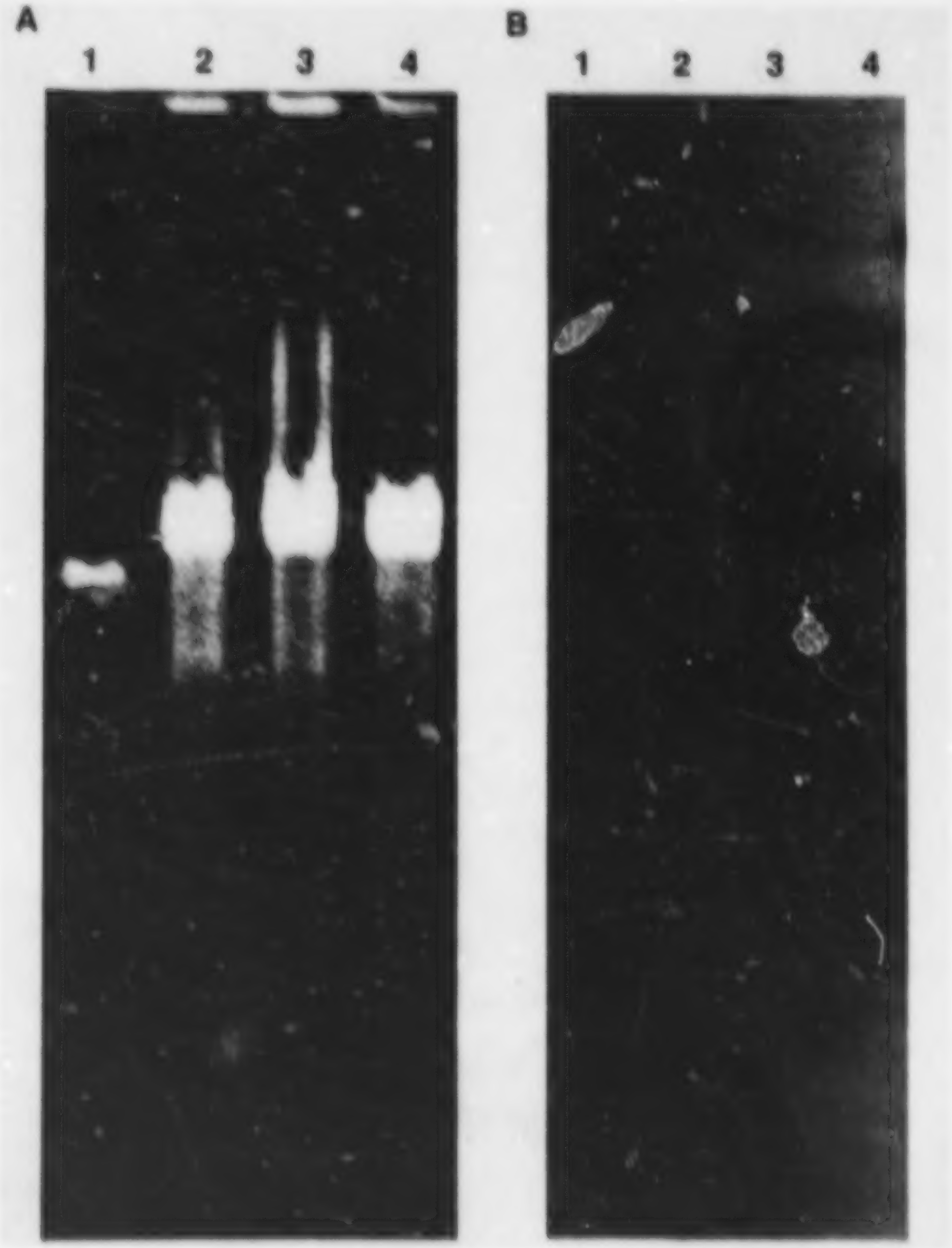

Fig. 4 Southern Blot Analysis of Undigested Phage and Chromosomal DNA. Undigested φAc1 virion DNA and undigested chromosomal DNA from strains CM9 (a sensitive, recipient cell), CM9A (a naturally isolated lysogen), and CM9/A4 (a lysogenic strain produced in the lab) were analyzed by Southern blot hybridization as described. Panel "A": Ethidium bromide stained gel; Panel "B": Autoradiograph of filter hybridized with ^{32}P labeled φAc1 virion DNA; lane 2: CM9 chromosomal DNA; lane 3: CM9/A4 chromosomal DNA; lane 4: CM9A chromosomal DNA.

Cloning and Characterization of *Thiobacillus ferrooxidans* Genes Involved in Sulfur Assimilation

Ilona J. Fry and Emilio Garcia

Biomedical Sciences Division,
L-452 Lawrence Livermore National Laboratory Livermore, CA 94551

ABSTRACT

The sulfate activating locus from *Thiobacillus ferrooxidans* has been cloned and expressed in *Escherichia coli*. The genes encoding ATP sulfurylase (ATP: sulfate adenylyl transferase, EC 2.7.7.4) and APS kinase (ATP: adenosine 5'-phosphosulfate 3'- phospho transferase, EC 2.7.1.25) have been isolated from a *T. ferrooxidans* genomic library constructed in pBR322. The approach used involved the utilization of mutants of E.coli defective in specific steps of the sulfur assimilatory pathway to isolate and identify, by genetic complementation, the corresponding genes and gene products in *T. ferrooxidans*.

In *E. coli*, the genes of sulfur assimilation are found in a locus mapping at 59 minutes of the chromosome. Included here are a cluster of genes organized in an operon designated *cys*DCHIJ. The proteins encoded by these genes include: ATP sulfurylase, APS kinase, PAPS reductase and sulfite reductase respectively. A plasmid designated pTFcys 13 which contains a 4.7 kb *T. ferrooxidans* insert was found to complement both *cysD*- and *cysC*- *E. coli* mutants. pTFcys 13 did not complement *E.coli* mutants carrying either *cys I*, *cysJ* or *cysH* defective genes. The genetic organization of the sulfate reducing locus has been inferred from deletion analysis of the insert in pTFcys13 coupled to complementation studies. Most deletions obtained led to a complete loss of ATP sulfurylase activity pointing to the possibility that more than one gene may encode this enzyme and thus, to its probable multimeric nature. The multimeric make-up of the sulfurylase was verified by *in vitro* transcription-translation studies with the *cysD*-complementing plasmid that indicated the enzyme is made up of two non-identical subunits with an estimated size of 36 and 62kD.

LE CLONAGE ET LA CARACTÉRISATION DES GÈNES DE *THIOBACILLUS FERROOXIDANS* IMPLIQUÉS DANS LE PROCESSUS D'ASSIMILATION DU SOUFRE

Ilona J. Fry et Emilio Garcia

Division des Sciences Biomédicales, L-452
Laboratoire National de Lawrence Livermore
Livermore, CA 94551, É.-U.

RÉSUMÉ

Le locus responsable de l'activation du sulfate chez *Thiobacillus ferrooxidans* a été cloné et exprimé dans *Escherichia coli*. Les gènes qui codent pour l'ATP sulfurylase (ATP : sulfate adénylyl transférase, EC 2.7.7.4) et ceux qui codent pour l'APS kinase (ATP: adénosine 5'-phosphosulfate 3'-phospho tranférase, EC 2.7.1.25) ont été isolés à partir d'une banque de gènes de *T. ferrooxidans* construite avec pBR322. La stratégie employée consistait à utiliser des mutants de *E. coli* défectueux pour des étapes spécifiques de la voie d'assimilation du soufre pour isoler et identifier, par complémentarité génétique, les gènes correspondants et les produits de ces gènes chez *T. ferrooxidans* .

Chez *E. coli*, les gènes responsables de l'assimilation du soufre sont retrouvés dans un locus situé à 59 minutes sur le chromosome. On y retrouve un amas de gènes qui forment un opéron désigné *cys* DCHIJ. Parmi les protéines codées par ces gènes se trouvent : l'ATP sulfurylase, l'APS kinase, la PAPS réductase et la sulfite réductase. Un plasmide désigné pTFcys^{13} qui comprend une insertion de *T. ferrooxidans* de 4.7 kb s'est avéré être complémentaire aux mutants de *E. coli* cysD$^-$ et cysC$^-$. Aucune complémentarité n'a été observée entre pTFcys 13 et des mutants de *E. coli* porteurs des gènes *cysI*, *cysJ* ou cysH défectueux. L'organisation génétique du locus responsable de la réduction du sulfate a été déduite à partir de résultats obtenus dans des tests de délétion dans l'insertion chez pTFcys 13 et des tests de complémentarité. La plupart des délétions obtenues ont mené à une perte totale de l'activité de l'ATP sulfurylase, indiquant que peut-être plus d'un gène code pour cet enzyme qui serait alors en toute probabilité un multimère. La composition multimérique de la sulfurylase a été vérifiée par des études de transcription-traduction *in vitro* avec le plasmide complémentaire de *cysD*. L'enzyme est composé de deux sous-unités non-identiques de dimensions estimées à 36 et 62kD.

INTRODUCTION

The sulfur metabolism of *T.ferrooxidans* constitutes an interesting area of study. Not only can this organism oxidize a variety of sulfur compounds in energy yielding reactions, but it is also capable of reducing sulfate in assimilatory fashion through a pathway organized to incorporate sulfur atoms into the sulfur-containing amino acids cysteine and methionine. Thus, the *T. ferrooxidans* cell must posses the potential and the capability to regulate the synthesis of enzymes involved in diametrically opposed chemical reactions. Sulfate production in one case, and sulfate reduction in the other (figure 1*A* and *B*). Although a great deal is known about the nature of the biochemical steps involved in the oxidation of sulfur compounds in *T. ferrooxidans* (Silver and Lundgren, 1968 *a* and *b*), scant information is available on the sulfate assimilatory pathway.

Sulfate is taken up and assimilated by a variety of organisms to form the sulfur-containing amino acids cysteine and methionine (Schiff and Hodson, 1973). The first enzyme in the sulfate assimilation pathway, ATP sulfurylase, catalyses the activation of intracellular sulfate by ATP.

$$\text{ATP} + \text{Sulfate} \longrightarrow \text{APS} + \text{PPi}^{1}$$

In *E.coli* the gene encoding this enzyme (*cysD*) is subject to regulation. When cysteine is provided in the growth medium, the synthesis of the enzyme is repressed whereas supplying the cells with a poor sulfur source, such as reduced glutathione or djenkolic acid, leads to its derepression (Jones-Mortimer, 1968; Kredich, 1971).

The second enzyme in this pathway, APS kinase, catalyses the phosphorylation of APS.

$$\text{APS} + \text{ATP} \longrightarrow \text{PAPS} + \text{ADP}$$

The gene (*cysC*), encoding the enzyme that catalyzes this step, is coordinately regulated with *cysD*. Both in *E.coli* and *Salmonella typhimurium*, these genes reside adjacent to each other in the chromosome and together with *cysH, cysI, cysJ*, make up one of several *cys* custers present in these organisms (Jones-Mortimer, 1973; Leyh *et al.*, 1988).

In this paper we present work which describe the isolation of genes involved in the sulfate assimilation of *T. ferrooxidans*, their genetic organization and the identification of their protein products.

METHODS

Materials and Methods

Bacterial Strains and plasmids are described in Table 1

Culturing Methods. T. ferrooxidans was grown using the 9K iron medium of Silverman and Lundgren (1959). The assay and media conditions used for the determination of ATP sulfurylase and ATP kinase enzymatic activities have been described (Hanna and Taylor 1988; Fry *et al.*1989).

Gene library Construction and selection of clones expressing ATP sulfurylase activity. The preparation of a T. ferrooxidans genomic library has been described previously (Fry *et al.*, 1988). Selection of plasmids from the library expressing ATP sulfurylase and APS kinase activity was carried out by genetic complementation of strains JM221 and JM81A, a *cysD-* and *cysC- E. coli* mutant respectively. The complementation studies were performed by transforming the appropriate strain with DNA

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[1] Abbreviations used: APS, adenosine 5'-phosphosulfate; PAPS, 3'phosphoadenosine 5'-phosphosulfate; PPi, inorganic pyrophosphate; CMM, Clostridium minimal medium

isolated from pools of the entire 3,000-member genomic library and selecting for simultaneous ampicillin resistance and cysteine independence.

DNA manipulations. Ligations, plasmid deletions and other DNA manipulations were carried out using standard techniques of recombinant DNA (Maniatis, 1985; and Perbal, 1988). *Nick translation.* The probe was labeled with ^{32}P-dCTP using a nick translation kit purchased from New England Nuclear. Prehybridization and hybridizations were carried out overnight at 65 C° essentially as described by Southern (1975).

In vitro transcription-translations. Cell-free transcription translation of plasmids pTFcys13, pBScys13Δ *Hind*III and its respective derivatives was done using a Prokaryotic *In Vitro* Transcription-Translation kit purchased from Amersham.

RESULTS

Complementation of mutants and identification of genes. The approach used in these studies relied on genetic complementation of available mutants of *E. coli*, defective in specific steps of the sulfate assimilatory pathway, to isolate and identify the corresponding gene and gene products in *T. ferrooxidans*. Some of the *E. coli* mutants that were utilized for this study are shown in Table 2.

In *E.coli*, a number of the genes that are involved in sulfur assimilation are located at a locus mapping at 59 minutes on the chromosome. In particular, a cluster designated *cys*DNCHIJ that encodes the ATP sufurylase, APS kinase, PAPS reductase and sulfite reductase respectively, form part of an operon (Jones-Mortimer, 1973, Leyh *et al.*, 1988).

Using DNA from pooled clones from the *T. ferrooxidans* genomic library we transformed JM221, an *E.coli* mutant with a defective ATP sulfurylase, to cysteine independence. The selection was for those plasmids that could simultaneously confer the mutant ampicillin resistance and the ability to grow on plates containing sulfate as the only sulfur source. Two such plasmids were obtained and one of them, designated pTFcys13, was chosen for further studies. A map of pTFcys13 showing the restriction enzymes to compare sequences in the chromosome with those of pTFcys13. The results are shown in Fig. 3. As expected, only those lanes in which total digested *T. ferrooxidans* DNA is present showed hybridization bands with the 4.7 Kb BamHI insert probe from pTFcys13 (lanes 2, 3 and 7). No homology was observed between the probe and genomic DNA from JM221 (lane 11).

Since in other bacterial species the genes involved in sulfate assimilation occur as a cluster on a specific region of the chromosome (Jones-Mortimer, 1968; Leyh *et al.*, 1988), we tested pTFcys13 for the ability to complement a number of *E.coli* mutants defective in the sulfur assimilatory pathway. Mutants used for this purpose included: JM246 (*cysI*), AT2427 (*cysJ*), JM81A (*cysC*) and DG37 (*cysA*). In addition to JM221, only JM81A yielded recombinants in which sulfate utilization had been restored. Therefore, it was assumed that besides the *cysD* gene, the *cysC* gene, encoding the APS kinase was present on the pTFcys13 insert. The sulfite reductase gene, that in the *E. coli cys* operon follows the *cysC*.

In vivo complementation and growth studies. The growth properties and the levels of ATP sulfurylase in response to exogenous sulfur source as studied in the JM221 mutant carrying pTFcys13. As shown in figure 4, panel B, pTFcys13 restores JM221 the ability to grow in sulfate minimal medium. In fact, JM221/pTFcys13 can grow in this medium at the same rate as if the medium is supplemented with cysteine or reduced glutathione. In contrast, if JM221 carries the pBR322 vector only, no restoration of sulfate utilization is observed (panel A). The putative regulation of the cloned *T. ferrooxidans* genes has been examined by measuring the levels of ATP sulfurylase activity conferred by pTFcys13 were unaffected by the sulfur source present in the growth medium. Thus, in contrast to that of *E. coli*, the cloned ATP sulfurylase from *T. ferrooxidans* does not seem to be regulated by the level of intracellular cysteine or reduced glutathione.

Genetic organization and gene products encoded from the T. ferrooxidans cys locus. To ascertain the genetic organization of the *cysD* and *cysC* loci present in pTFcys13 a number of deletions extending into the insert region of this plasmid were constructed and examined for their complementation pattern (Fry et al 1989). Only a deletion that extended from the HindIII site of the pBR322 vector to the HindIII site 1kb into the insert (figure 2), was found to retain the ability to complement JM221 (*cys* D) while becoming unable to complement JM81A (*cys* C). This was the first indication of a possible three-gene arrangement for this locus and suggested that the ATP sulfurylase might be encoded by more than one gene. Evidence for this genetic organization and for the fact that two gene products are being encoded by the *cysD* locus was obtained by subcloning the fragment remaining in the pTFcys13Δ *Hind*III plasmid into the pBS- cloning vector (Stratagene, La Jolla, CA.). The resulting plasmid, pBScys13Δ *Hind*III which lacked APS kinase-complementing activity by virtue of a deletion extending into the *cysC* gene, was used for *in vitro*, protein synthesis analysis of the *cysD* region. The deletion plasmid was digested with a series of restriction enzymes and the resultant linear DNA fragments (figure 5) were used to direct an *in vitro* transcription-translation system that enables the identification of polypeptides made from a DNA template. Two unique products not present in the pBR322 control were readily identified by auoradiography: a 62 and 36Kd polypetide respectively (indicated by arrows in Figure 6.). It was noted that the *EcoRI*-treated fragment gave rise to a truncated 61Kd peptide was completely abolished (lane 4). By correlating the peptides obtained from the various restriction fragments from pBScys13D*Hind*III with the parallel complementation and deletion studies, it was possible to infer the order of the genes of this locus and permitted the identification of the respective polypeptides that they encode. Thus, it was determined that the *cysD* gene, the first gene of this three-gene cluster, encodes the 62 Kd polypeptide (CysD product). While the *cysN* gene, which lies between *cysD* and the distal *cysC* gene, encodes the 36 Kd polypeptide (CysN product). This finding established the multimeric make up of the *T. ferrooxidans* sulfurylase. A summary depicting the gene arrangement, the corresponding peptides derived from this locus and a comparison between the analogous regions of *E.coli* and *T. ferrooxidans* is shown in figure 6.

CONCLUSIONS

Confirmation of the presence of ATP sulfurylase and APS kinase by this work supports the notion that sulfate assimilation to form cysteine and methionine operates through the *cys* pathway in *T. ferrooxidans*. Under proper growth medium conditions, *T. ferrooxidans* oxidizes inorganic sulfur compounds such as sulfur, tetrathionate and thiosulfate to obtain metabolic energy in the form of ATP. Both the dissimilatory and assimilatory pathway appear to have APS as an intermediate. That these two diametrically opposing reactions (which could easily lead to a futile cycle) could be occurring simultaneously has been questioned (Tuovinen *et al.*, 1975). Kelley *et al.*, (1976) determined that $^{35}SO_4$ was only taken up and assimilated when the growth medium for *T. ferrooxidans* was ferrous sulfate-based. However, these investigators partially purified an active ATP sulfurylase form both thiosulfate and ferrous sulfate grown cell whose levels did not appear to vary in response to the nature of the medium. They thus, concluded that the synthesis of ATP sulfurylase was constitutive but that it did not fulfill an assimilatory function when cells were grown on thiosulfate medium. Rigorous studies of the regulation of these genes cannot be carried out in a heterologous host such as *E. coli*, nevertheless, the fact that the synthesis of this enzyme, when produced in *E. coli*, was not susceptible to regulation by the levels of exogenous cysteine is consistent with a pattern of constitutive synthesis.

That the genes involved in the assimilation of sulfate are arranged in an operon-like structure in *T. ferrooxidans* was not entirely unexpected since other genes such as the nitrogen fixing (*nif*) genes (Pretorious *et al.*, 1986), the *gln*ALG genes (Barros *et al.*, 1985) and the genes of the ribosomal operon (Venegas *et al.*, 1988) also show this arrangement in *T. ferrooxidans*. It is nevertheless interesting to note the fact that organisms as phytogenetically different as *E. coli* and *T. ferrooxidans* seem to posses gene organizations so similar to one another.

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TABLE 1

Bacterial Strains Used in This Study

Strains	Genotype	Source of Reference
HB101	*pro-leu thi- lacY hsdR- endA- rpsL20 ara-14 galK2 xyl5 mtl- 1 supE44 recA-*	Bachmann and Bolivar, 1979
JM221	*cysD91 pro50 his97 trp-74 argA- ilvA lac- gal- galT47 xyl- mtl- mal- strA- tsx-*	received as CGSC 5745 from B. Bachmann
JM246	*cys153 lambda- 1N(rrnD-rrnE)1*	received as CGSC 4747 from B. Bachmann
AT2427	*sysJ43 relA1 thi-1 lambda- spot1*	received as CGSC 4502
JM96	*cysH56 thr-1 leuB6 trp- argH1 thi-1 ara-13 laY1 gal-6 malA1 xyl-7 mtl-2 strA9 tonA2 supE44 lambda- lambda, R*	received as CGSC 5746 from B. Bachmann
JM81A	*cysC*	received as CGSC 5744 from B. Bachmann
T. ferrooxidans (ATCC 218361)	wild-type	ATCC

TABLE 2

Pattern of Complementation of pTFcys13 on Various *E.coli* Mutants Defective in the Sulfate Assimilatory Pathway

Mutant		Response*
JM221	(*cys* D)	+
JM81A	(*cys* C)	+
AT2427	(*cys* J)	—
JM246	(*cys* I)	—
JM96	(*cys* H)	—
DG37	(*cys* A)	—

* A positive response denotes growth with sulfate as sulfur source.

TABLE 3

ATP Sulfurylase And Sulfite Reductase Activity In Crude Extracts of *E. Coli* And *T. Ferrooxidans*

	Specific Activity		
	ATP Sulfurylase pMole APS/min/mg	Sulfite Reductase nMole NADP/min/mg	
HB101/pBR322			
1.	SULFATE	210.0	8.78
2.	CYSTEINE	<0.3	<0.10
3.	RED.GSH	1260.0	6.10
JM221/pBR322			
4.	RED.GSH	<0.3	3.57
JM221/pTFcys13			
5.	SULFATE	29.0	3.34
6.	CYSTEINE	21.6	<0.1
7.	RED.GSH	21.1	2.87
*T. ferrooxidans**	3.3	1.9	

Cell extracts were assayed for ATP sulfurylase activity according to the method of Hanna and Taylor (1989) and for sulfite reductase activity using the method of Yoshimoto *et al.*(1971). Source of the crude extract were cells grown to mid-logarithmic phase in minimal medium containing 0.7 mM of the sole sulfur source indicated above (Fry *et al.*, 1988: Hanna and Taylor, 1989)

* Grown in Fe SO_4

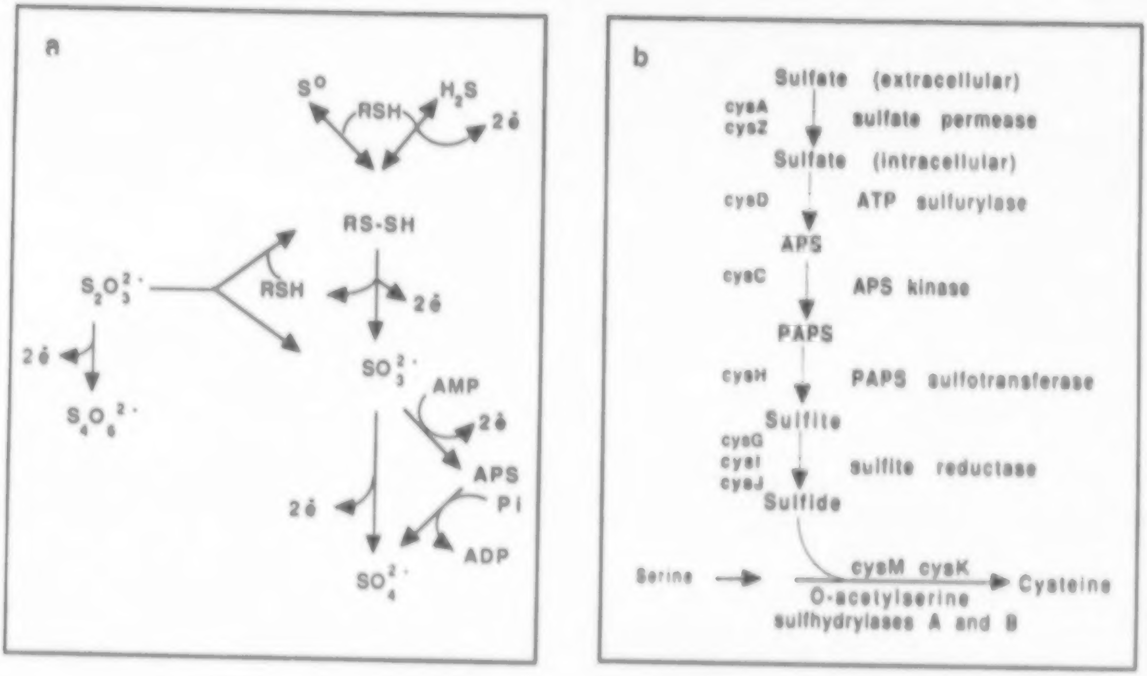

a
S°
RSH
H₂S
2ė
RS-SH
S₂O₃²⁻
RSH
2ė
2ė
S₄O₆²⁻
SO₃²⁻
AMP
2ė
APS
2ė
Pi
ADP
SO₄²⁻
b
Sulfate (extracellular)
cysA
cysZ
sulfate permease
Sulfate (intracellular)
cysD
ATP sulfurylase
APS
cysC
APS kinase
PAPS
cysH
PAPS sulfotransferase
Sulfite
cysG
cysI
cysJ
sulfite reductase
Sulfide
Serine
cysM cysK
O-acetylserine sulfhydrylases A and B
Cysteine

Fig. 1 Generalized Scheme of Microbial Sulfur Metabolism. (A) Proposed pathway for the oxidation of inorganic sulfur compounds in the thiobacilli (modified from Siegel, 1975). (B) Pathway of sulfate assimilation in enterobacteriace. The genetic loci indicated for each enzymatic step are those defined for *S. typhimurium* (modified form Kredich, 1987).

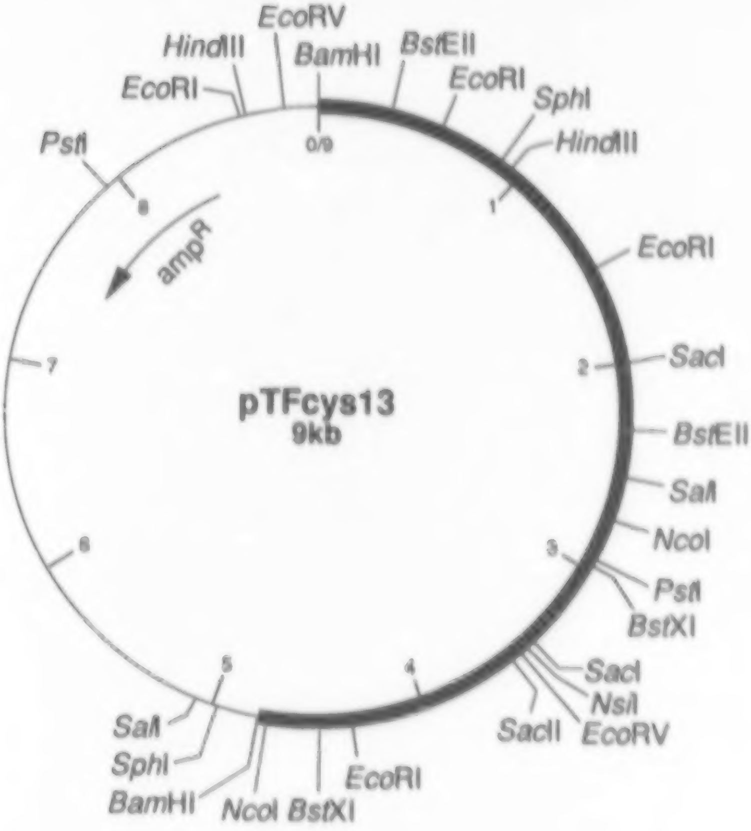

EcoRV
HindIII
BamHI
BstEII
EcoRI
EcoRI
SphI
HindIII
PstI
0/9
1
8
ampR
EcoRI
2
SacI
7
pTFcys13
9kb
BstEII
SalI
NcoI
6
3
PstI
BstXI
5
4
SacI
NsiI
SacII
EcoRV
SalI
SphI
BamHI
NcoI
BstXI
EcoRI

Fig. 2 Physical Map of pTFcys13. Restriction endonuclease sites within the 4.7 KB *BamHI* insert (thick line) of *T. ferrooxidans* are shown. The direction of transcription of the b-lactamase gene of the pBR322 vector is illustrated by the arrow.

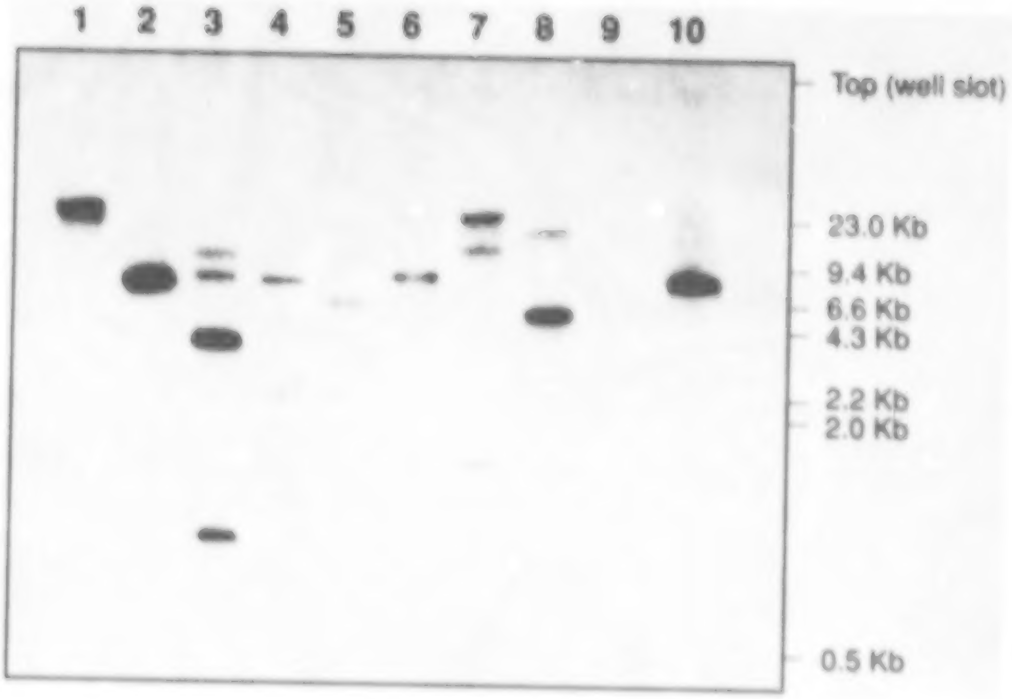

1
2
3
4
5
6
7
8
9
10
Top (well slot)
23.0 Kb
9.4 Kb
6.6 Kb
4.3 Kb
2.2 Kb
2.0 Kb
0.5 Kb

Fig. 3 Southern blot analysis of pTFcys13. Total cellular DNA from *T. ferrooxidans* and *E. coli* was digested to completion with the restriction enzymes indicated and separated by electrphoresis on a 0.8% agarose gel. After transfer to nitrocellulose, the DNA was hybridize with the $[^{32}P]$ labelled BamHI insert from pTFcys13. Lane 1, *T. ferrooxidans* (TF) uncut; lane 2, TF *BamHI*; lane 3, TF *EcoRI*; lane 4, TF *EcoRV*; lane 5, TF *HindIII*; lane 6, TF *PstI*; lane 7, TF *SacI*; lane 8, TF *SalI*; lane 9, JM221 *BamHI*; lane 10, pTFcys13 *BamHI*.

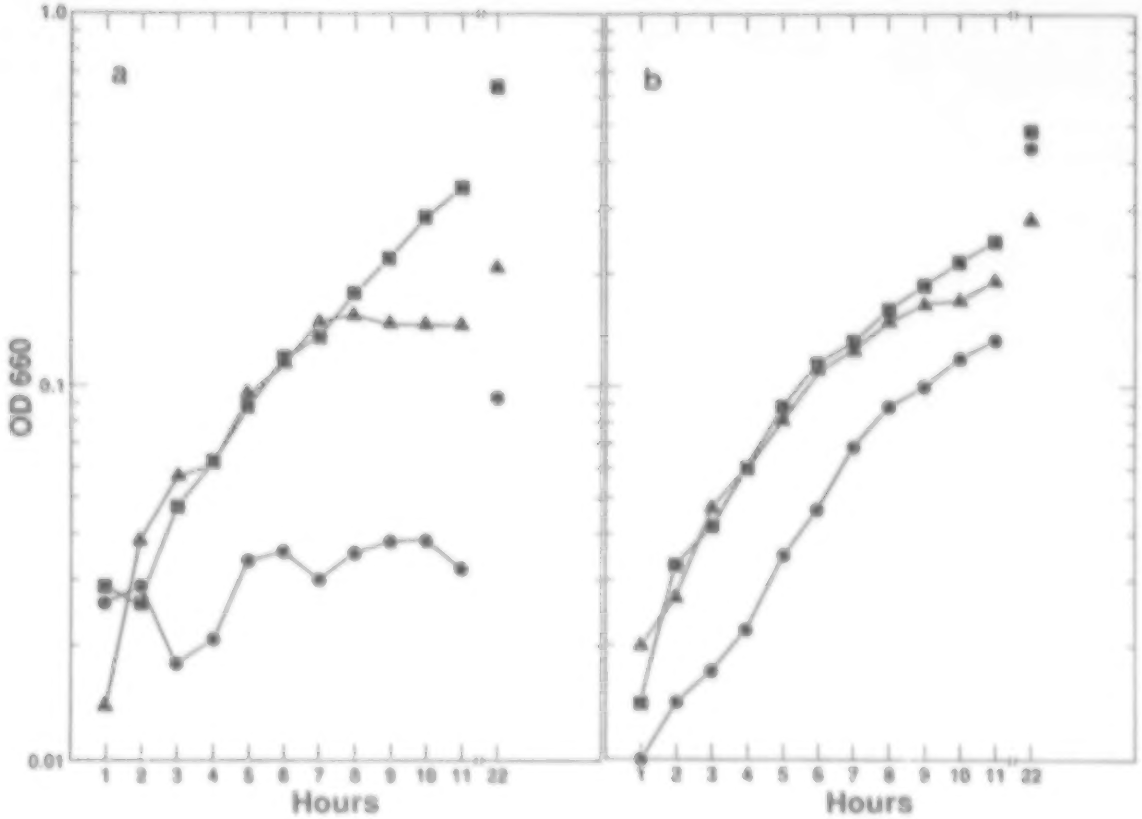


Fig. 4 Growth curves of JM221/pBR322 (panel A) and JM221/pTFcys13 (panel B). Cells grown in sulfate minimal medium (•); cysteine-supplemented medium (▲) and in medium supplemented with glutathione (▲).

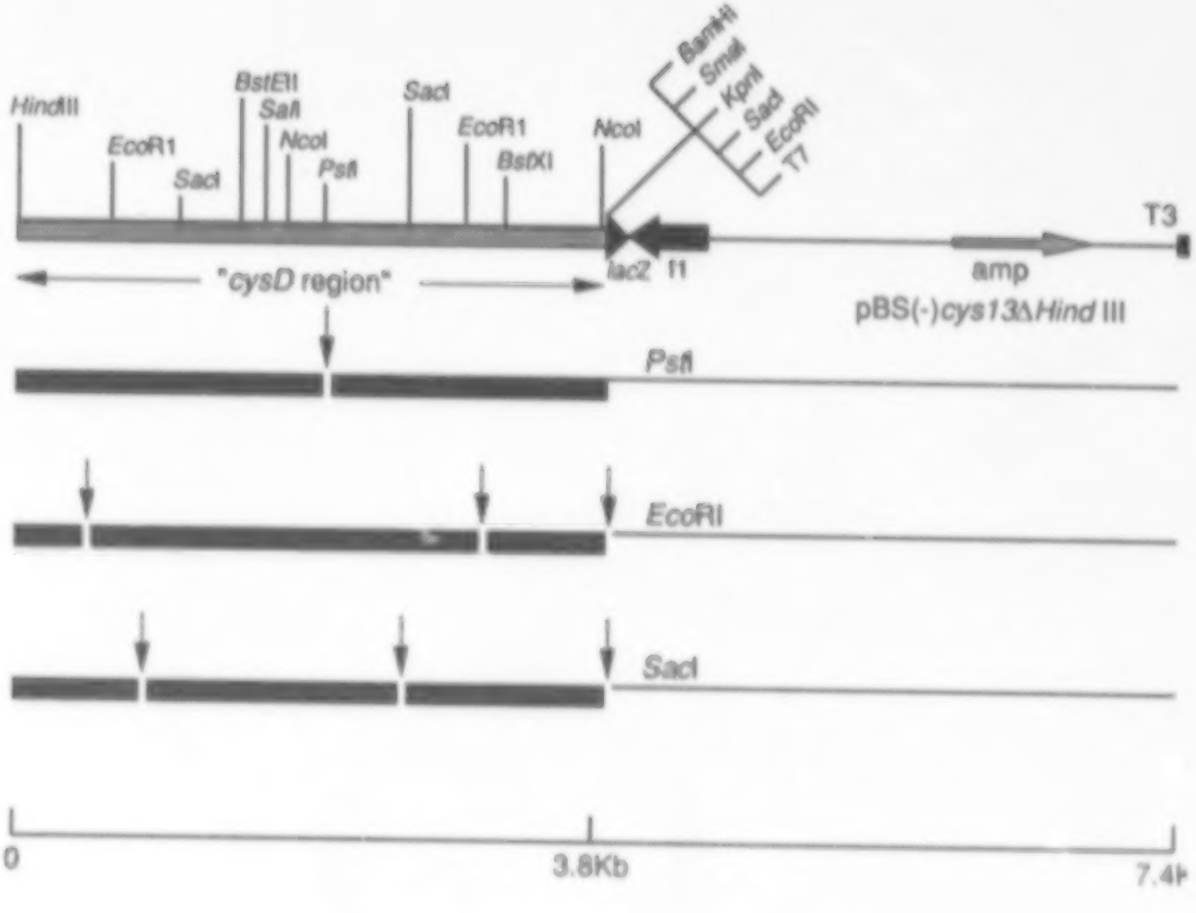

HindIII
EcoR1
SacI
BstEII
SalI
NcoI
PstI
SacI
EcoR1
BstXI
NcoI
BamHI
SmaI
KpnI
SacI
EcoRI
T7
T3
"cysD region"
lacZ
f1
amp
pBS(-)cys13ΔHind III
PstI
EcoRI
SacI
0
3.8Kb
7.4k

Fig. 5 Map restriction endonuclease-generated fragments used in the synthesis of proteins in an *E. coli* DNA-directed cell-free system. Fragments indicated by the arrows were obtained by linearizing pBScys13DHindIII with *PstI*, *EcorI* and *SacI* respectively.

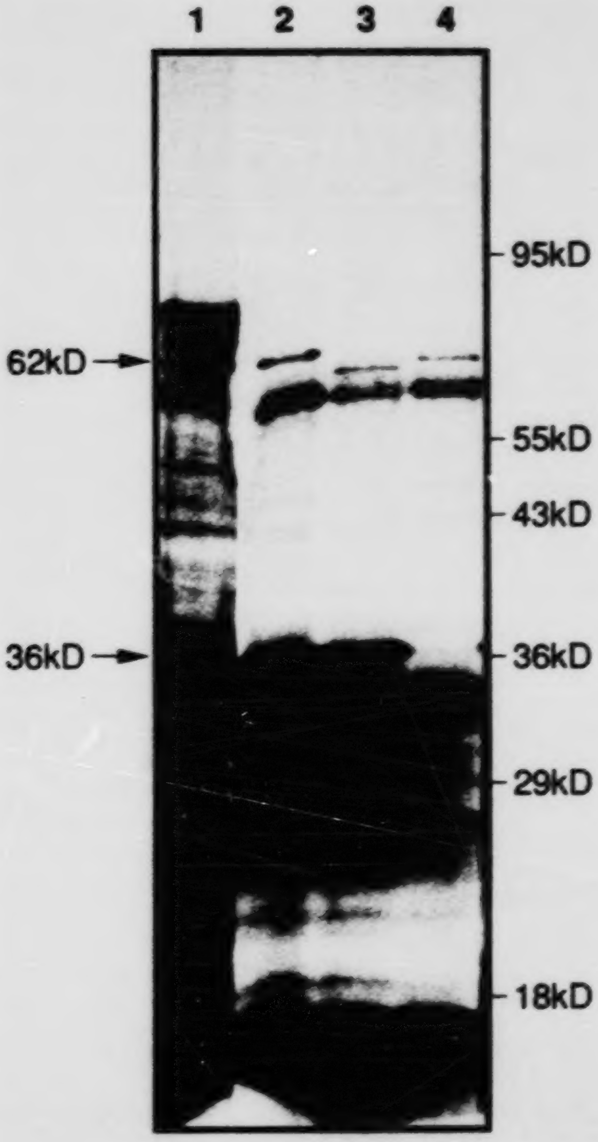
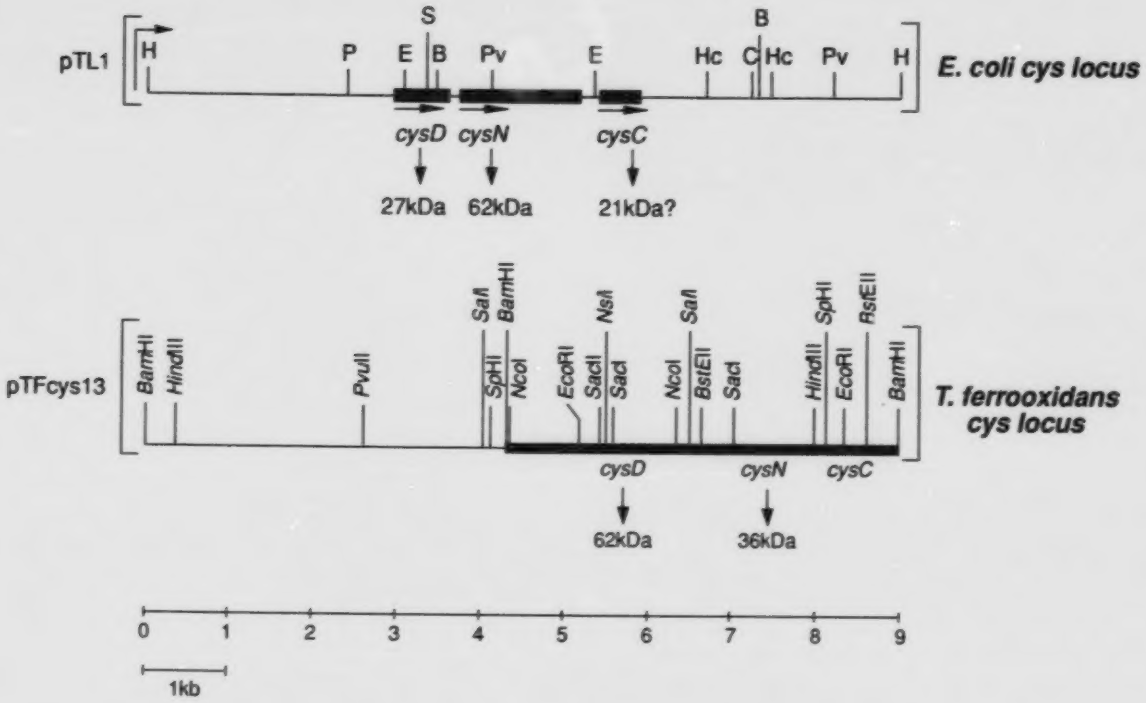

1
2
3
4
62kD
36kD
95kD
55kD
43kD
36kD
29kD
18kD

Fig. 6 DNA-dependent cell-free synthesis of cloned proteins. The gene products labeled with [^{35}S] methionine were analyzed on SDS-polyacrylamide gel electrophoresis. DNA template was linearized with restriction enzymes as indicated. Lane 1, pBScys13Δ *Hind* III uncut; lane 2, pBScys13 Δ *Hind* III/*Pst*I; lane 3, pBScys13Δ *Hind* III/*EcoR*I; lane 4, pBScys13Δ *Hind* III/ *Sac*I.


pTL1
H
P
E
S
B
Pv
E
Hc
B
C
Hc
Pv
H
E. coli cys locus
cysD
cysN
cysC
27kDa
62kDa
21kDa?
pTFcys13
BamHI
HindIII
PvuII
SalI
SpHI
BamHI
NcoI
EcoRI
SacII
NsiI
SacI
NcoI
SalI
BstEII
SacI
HindIII
SpHI
EcoRI
BstEII
BamHI
T. ferrooxidans
cys locus
cysD
cysN
cysC
62kDa
36kDa
0
1
2
3
4
5
6
7
8
9
1kb

B-EG-6 (10-2-89) FH

Fig. 7 Physical map and polypeptide expression in the *T. ferrooxidans* cloned insert and its comparison to the analogous cloned region from *E. coli* (Leyh, 1988). The approximate location of the region of DNA encoding for the protein products was obtained from experiments describe in the text. The direction of transcription has not been determined but it is presumed to proceed from *cysD* to *CysC*.

COMPARATIVE ANALYSIS OF *THIOBACILLUS FERROOXIDANS* STRAINS

by

P. VALENTI[*], *M. POLIDORO,*
V. BUONFIGLIO, P. VISCA, N. ORSI

Istituto di Microbiologia, Universita' di Roma "La Sapienza", P.le A. Moro 5, 00185 Roma.

ABSTRACT

Thiobacillus ferrooxidans strains isolated in different environments and geographical areas have been analyzed for their plasmid content. A 20 Kb plasmid (pTFO) has been detected in the majority of the examined strains. The physical map of pTFO was virtually identical to that reported by Rawlings *et al.* (1984) for pTF35. Preliminary results suggest that pTFO does not encode for metal-resistance and its functions cannot be assigned so far. The analysis of whole and outer membrane proteins revealed a high degree of similarity among strains with identical plasmid profiles and noticeable differences among strains with different plasmid patterns.

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[*] Istituto di Microbiologia, Universita' di Napoli, Larghetto S. Aniello a Caponapoli 80126 Napoli, Italy.

ANALYSE COMPARATIVE DES DIFFÉRENTES SOUCHES DE *THIOBACILLUS FERROOXIDANS*

*P. Valenti *, M. Polidoro,*
V. Buonfiglio, P. Visca et N. Orsi

Institut de Microbiologie, Université de Rome "La Sapienza", Rome

RÉSUMÉ

Les plasmides contenus dans des souches de *Thiobacillus ferrooxidans* isolées dans divers milieux et différentes régions géographiques ont fait l'objet d'analyses. Dans la plupart des souches étudiées, on a décelé un plasmide (pTFO) mesurant 20 kb. La carte génétique de pTFO est presque identique à celle qui a été dressée par Rawling et al (1984) pour pTF35. Les résultats préliminaires donnent à penser que pTFO ne détermine pas le caractère de résistance aux métaux; ses fonctions ne sont pas encore connues. L'analyse des protéines de la membrane externe montre qu'il existe une grande ressemblance entre les souches qui présentent le même profil de plasmides, mais de grandes différences entre celles qui ont des profils différents.

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* Institut de Microbiologie, Université de Naples, Naples, Italie

INTRODUCTION

Thiobacillus ferrooxidans is a Gram-negative, acidophilic, chemoautotrophic bacterium deriving the energy for its growth from the oxidation of ferrous to ferric ions and of reduced sulphur compounds to sulphate. As a consequence of these peculiar metabolic features, this bacterium is the most important microorganism involved in bioleaching of low grade ores (Torma, 1988). Although the metabolism of *T. ferrooxidans* is fairly well known, its genetic analysis is at an early stage of investigation. *Thiobacillus* spp. can grow in environments characterized by high concentrations of several metal ions, generally toxic to other bacteria. As it has been shown that metal-resistance is often plasmid-encoded (Chopra, 1975; Summers *et al.*, 1978; Summers and Silver, 1978); it might also be possible that the *T. ferrooxidans* genes involved in detoxification of metals are plasmid-borne. The characterization of such plasmid markets in *T. ferrooxidans* has the potential for the production of genetically manipulated bacteria with enhanced leaching capabilities.

Plasmid occurrence in this species has been shown since 1980 (Mao *et al.*, 1980) but their function is so far only partially known. In 1984, Rawlings *et al.* demonstrated the expression of the origin of replication from a *T. ferrooxidans* plasmid in *Escherichia coli* and successively the mobilization region of a *T. ferrooxidans* plasmid was also identified and expressed in *E. coli* (Rawlings and Woods, 1985). These observations indicate that at least some signals for gene expression may be similar in the acidophilic chemolitotrophic *T. ferrooxidans* and in heterotrophic bacteria.

In our research, twelve strains of *T. ferrooxidans* isolated from different parts of the world have been examined for plasmid content, metal-resistance and other biochemical characters, with the aim to correlate plasmid profiles with relevant phenotypic traits.

MATERIALS AND METHODS

Bacterial Strains Five of the twelve *T. ferrooxidans* strains used in this work have been isolated from different environments within the same geographical area on the outskirts of Rome (Oriolo Romano) and characterized as previously reported (Visca *et al.*, 1989); strain TFOB was isolated from the undergrowth in Sterpeto bush, TFOF from an acid stream (pH 5.2) associated with jarosite deposits (Biscione stream), TFOS and TFMSR from a sulphuric spring (pH 4.5), and TFVS from the Mignone River approximately 5 km downstream from the sulphuric spring. A detailed map of the region is presented in Fig. 1. The other seven strains have been isolated from various ores in different parts of the world: strains TFNM1 and TFNM3 have been isolated from chalcopyrite ($CuFeS_2$) mines in Socorro, New Mexico, USA; strain TFP4 from the chalcopyrite mine of S. Valentino di Predoi, Bolzano, Italy; strain TFMV from the mixed galena (PbS)-sphalerite (ZnS) mine of Montevecchio, Cagliari, Italy; strain TFFC from the mixed pyrite (FeS_2)-chalcopyrite mine of Fenice Capanne, Grosseto, Italy; strain TFGO2 from the mixed millerite (NiS)-cobalt sulfide-pyrite ore of Genna s'Olioni, Cagliari, Italy; strain TFN11 from Bulgaria.

Media and Growth Conditions Strains were routinely cultivated in 9K liquid medium (Silverman and Lundgren, 1959) at 28°C in shaken flasks (250 rpm in a New Brunswick Model G25 orbital shaker). The isolation of single clones was performed on the solid medium TSM1 (Visca *et al.* 1989). Bacterial growth and iron oxidation were analyzed as previously described (Visca *et al.* 1983).

Cell lysis and Plasmid DNA Extraction Bacterial cultures in late stationary phase (about 96 hours old) were filtered on Whatman No 1 paper in order to remove iron precipitates. Bacteria were harvested by centrifugation (5000 g, 20 min., 4 C). Cells were washed twice in 1 mM H_2SO_4 (pH 2.5) and once in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0). Cells were lysed according to a modification of Birnboim procedure (1983); briefly, the lysozyme solution was substituted by the TSE buffer (25% sucrose, 50 mM Tris-HCl pH 7.5, 20 mM EDTA, pH 8.0) and the final lysozyme concentration was decreased from 1 mg/mL to 0.75 mg/mL. Cells deriving from 100-mL cultures were suspended in 250 ul of TSE buffer, and lysis was carried out at 0 C for 15 minutes. Furthermore, samples were kept in 0.2 N NaOH-1% SDS solution only for 3 minutes to minimize chromosomal DNA contamination. Contaminating RNA was removed by treatment with DNase-free RNase (Ribonuclease A, Sigma). In some cases, plasmid DNA was purified by ultracentrifugation to equilibrium in CsCl-ethidium bromide gradients according to Maniatis *et al.* (1982).

Preparation of Whole and Outer Membrane Proteins Whole proteins (WP) from *T. ferrooxidans* were prepared by lysing bacteria (about 10^9) cells) in 50 ul of 10 mM Tris, 5 mM EDTA, 0.5% SDS. To detect protease-resistant proteins, samples were supplemented with 0.8 ug/ul proteinase K (about 20 μg/mg of protein). Lyses and digestions were carried out for 2 h at 65°C and protein hydrolysis was terminated with 1 mM phenylmethylsulphonyl fluoride. Protein concentrations were determined by the Bradford method (1976).

Outer membrane proteins (OMP) were prepared according to a modification of the procedure described by Filip *et al.* (1973). Cells deriving from 400 mL cultures in late exponential growth phase were harvested by centrifugation (5000Xg, 20 min., 4°C), washed as previously described and suspended in 1 mL of 50 mM Tris pH 8. OM were disrupted by ten cycles of 1 min. sonication, cell debris removed by centrifugation for 15 min at 4500X g and supernatants were supplemented with 10 μg of the protease inhibitor phenylmethylsulphonyl fluoride. Cytoplasmic membranes were solubilized with 2% (wt/vol) of the ionic detergent N- lauroylsarcosine and OM collected by centrifugation at 33.000 g for 1 h at 4°C.

Gel Electrophoresis of DNA Plasmid DNA was resolved in 0.8-1% agarose (Low m_r, Biorad) gel electrophoresis (4 volt/cm) in Loening buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.8). Polyacrilamide gels (3.5%) prepared as described by Maniatis *et al.* (1982) were run in TBE buffer (89 mM Tris, 89 mM H_3BO_3 2.5 mM EDTA, pH 8.3) and used to detect low molecular weight DNA fragments. After electrophoresis the gels were strained in 0.5-1 μg/mL ethidium bromide and photographed with a Polaroid type 66S film and a yellow filter. Hind III, Hind III-Eco RI and Pst I lambda phage DNA digests were used as markers of known molecular weight.

Electrophoresis of Proteins Protein samples were resolved by polyacrylamide-sodium dodecyl sulphate (PAGE-SDS) gel electrophoresis as described by Laemmli (1970). The resolving gel consisted of 10% polyacrylamide (ratio monomer to dimer 44:0.3) in 0.75 M Tris pH 8.8 containing 0.2% SDS, with a 5% stacking gel in 0.25 M Tris pH 6.8 also containing 0.2% SDS. The electrophoresis buffer was 25 mM Tris pH 8.3 containing 20 mM glycine and 0.2% SDS. As molecular weight standards the following proteins were used: rabbit muscle phosphorilase b (97.4 Kdal), bovine serum albumin (66.2 Kdal), hen egg white ovalbumin (42.7 Kdal), bovine carbonic anhydrase (31.0 Kdal), soybean trypsin inhibitor (21.5 Kdal), hen egg white lysozime (14.4 Kdal). Gels were run 12 h at 15 μA and stained with Coomassie brilliant blue, de-stained and photographed.

Cleavage of DNA by Restriction Endonucleases Restriction enzymes were purchased from New England Biolabs and used according to the protocols described by the manufacturer.

Metal Ions Sensitivity Test Different concentrations of the metal salts $AgNO_3$, $Cd(NO_3)_2.4H_2O$, $NiSO_4.6H_2O$, $CuSO_4.5H_2O$, $NaAsO_2$, $Na_2HAsO_4.7H_2O$ and $SbCl_3$ were added to 9K liquid medium in order to determine the concentration of the toxic metal ions capable of inhibiting both ferrous iron oxidation and growth.

Electron Microscopy CsCl-purified plasmid preparations were spread on parlodion-coated grids, stained with uranyl acetate and shadowed with 80:20 Pt-palladium according to Kleinschmidt (1968). The grids were examined with a Zeiss model 9A electron microscope. The contour measurements from the electronmicrographs were carried out using pBR322 as internal standard.

RESULTS

Analysis of Plasmids In order to determine the plasmid patterns of the *T. ferrooxidans* strains used in this work, we developed a procedure for the isolation of plasmid DNA derived from the alkaline extraction method conceived by Birnboim in 1983. We have modified Birnboim's protocol by adding some preliminary steps required to remove ferric iron precipitates formed during growth of *T. ferrooxidans* in 9K liquid medium and to neutralize the acid pH of solution used in the first extraction step (see *Materials and Methods*). The method is simple, rapid and provides reproducible results; the plasmid DNA yielded is mostly in closed circular form and pure enough to be digested by restriction endonucleases.

Figure 2 shows plasmids from strains TFOB, TFOF, TFOS, TFVS and TFMSR. Although isolated from different environments in the same geographical area, all these strains harbor a 20 kilobases (Kb) plasmid; strains TFOB, TFOF and TFOS contain also a smaller plasmid of about 9 Kb. Two different circular covalently closed DNA molecules of about 19.7 and 9.3 Kb were also detected by electron microscopy of CsCl-gradient purified DNA samples of strains TFOB (Fig. 3) and TFOF and TFOS (not shown)). Plasmids have been compared by digestion with the restriction endonucleases Bam HI and Hind III. Fig. 4 shows identical restriction patterns for the 20 Kb plasmid, with common additional DNA fragments detectable only in strains TFOB, TFOF and TFOS resulting from the 9 Kb plasmid digestion. The restriction map of the 20 Kb plasmid, which we termed pTFO, has been obtained; this plasmid contains an unique Hind III site, four Pst I sites, four Bam HI sites, five Sal I sites and seven Eco RI sites (not shown in the map) (Fig. 5). Most of these restriction sites are concentrated in a 10 Kb region of pTFO.

Occurrence of pTFO in T. ferrooxidans Strains from Different Parts of the World The plasmid content of some strains isolated in different parts of the world has also been characterized. It has been demonstrated that strains TFNM1, TFNM3 and TFP4 harbor pTFO. Strains TFFC, TFN11 and TFMV have very similar plasmid profiles which differ from that of strain TFG02, but they all lack pTFO (Fig. 6).

Metal Resistance An attempt has been made to correlate the presence of plasmids in *T. ferrooxidans* with the resistance to some toxic metal ions.

On the basis of the results obtained, it is not yet possible to assign any function to *T. ferrooxidans* plasmids. As a matter of fact, only minor differences in metal resistance have been noted among strains with different plasmid contents.

Comparison of Whole and Outer Membrane Proteins Among T. ferrooxidans Strains We characterized our *T. ferrooxidans* strains at protein level in order to determine whether strains with identical plasmid content showed a similar profile of whole and outer membrane proteins. Fig. 7 shows the profile of WP and OMP from strains TFOB, TFOF, TFOS, TFVS, TFMSR, TFMV, TFFC and TFG02. Strains isolated in the same geographic area on the outskirts of Rome have the same pattern of WP and OMP which was also identical to that of strains TFNM1 and TFNM3 isolated in Socorro, New Mexico and TFP4 isolated in Predoi mine, Italy (not shown in Fig. 7); other strains such as TFFC and TFMV, isolated from different areas, share noticeable similarities at protein level with the above strains (except for the absence of a 57 Kdal cytoplasmic protein and a 77 Kdal OMP) from which however they differ at plasmid level. Strain TFG02, characterized by a unique plasmid profile, is also significantly different in WP and OMP patterns from all the other strains. The most striking differences consist in the lack of the 37.5 and 50 Kdal major OMPs observed in the other strains and in the presence of peculiar protein bands; among these, the 39 Kdal OMP displays a marked resistance to proteolysis by proteinase K as shown in Fig. 8.

CONCLUSIONS

The results obtained show that seven out of the twelve *T. ferrooxidans* strains examined harbor a 20 Kb plasmid which we have called pTFO.

The physical map of this plasmid was determined by restriction endonuclease digestions and it is identical to the one reported by Rawlings *et al.* (1984) for pTF35. Plasmid pTFO has been found in *T. ferrooxidans* strains isolated from different environments and very distant geological areas; these strains have been subcultured for years without any selective pressure, thus indicating that pTFO is stably conserved for many generations.

By testing the growth and iron oxidation of the strains in 9K liquid medium supplemented with different metal ions it was not possible to correlate the presence of pTFO with the metal- resistance. All of the strains, in spite of their different plasmid patterns, were capable of resisting similar concentrations of toxic metals. This observation suggests the existence of a common mechanism of metal-resistance which could be based on membrane permeability and/or oxido-reductase enzymes present on the bacterial surface.

On the contrary, the OMP and WP profiles varied significantly among groups of strains, reflecting differences noticed in plasmid patterns.

In conclusion, the results obtained from plasmid and protein analysis indicate that strains TFOB, TFOF and TFOS could be considered as the same strain colonizing various environments in the region of Oriolo Romano and this observation could be also extended to strains TFVS and TFMSR. It was very interesting to note that strains TFNM1 and TFNM3, although isolated very far from Italy, appeared identical to the Italian strains TFVS and TFMSR, both plasmid and protein profiles.

ACKNOWLEDGEMENTS

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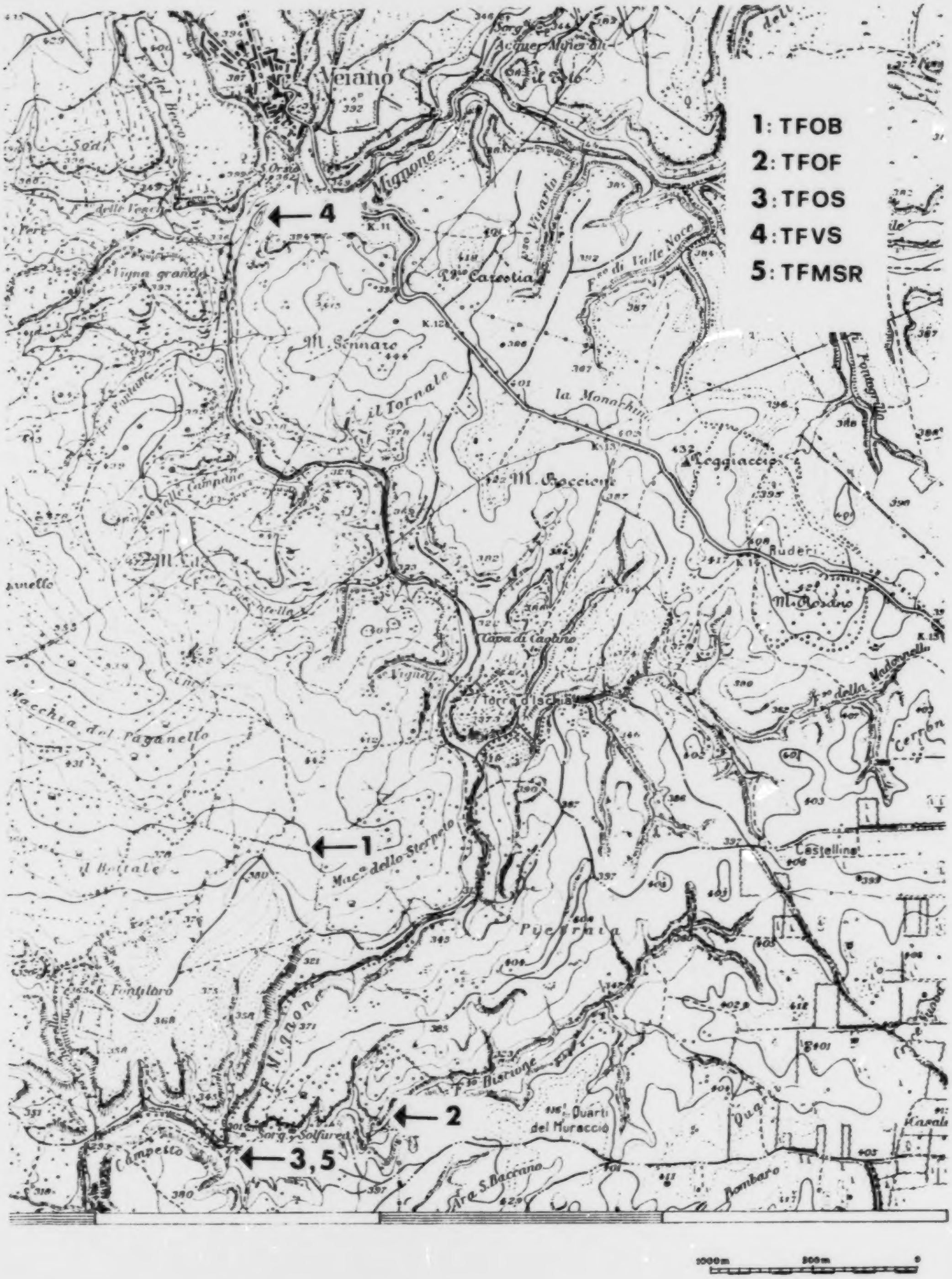
1: TFOB
2: TFOF
3: TFOS
4: TFVS
5: TFMSR
← 4
← 1
← 2
← 3,5
Veiano
Mignone
M. Sennaro
il Tornale
la Monarchia
Leggiaccio
M. Bocciona
Ruderi
M. Rosano
Torre d'Ischia
Macchia del Paganello
Macᵃ dello Sterpeto
Castellina
Quarti del Muraccio
Sorg.ᵉ Solfurea
Campetto
C. Fontiloro
Casale
1000m
500m
0

Fig. 1 Map of the geographical area of Oriolo Romano.

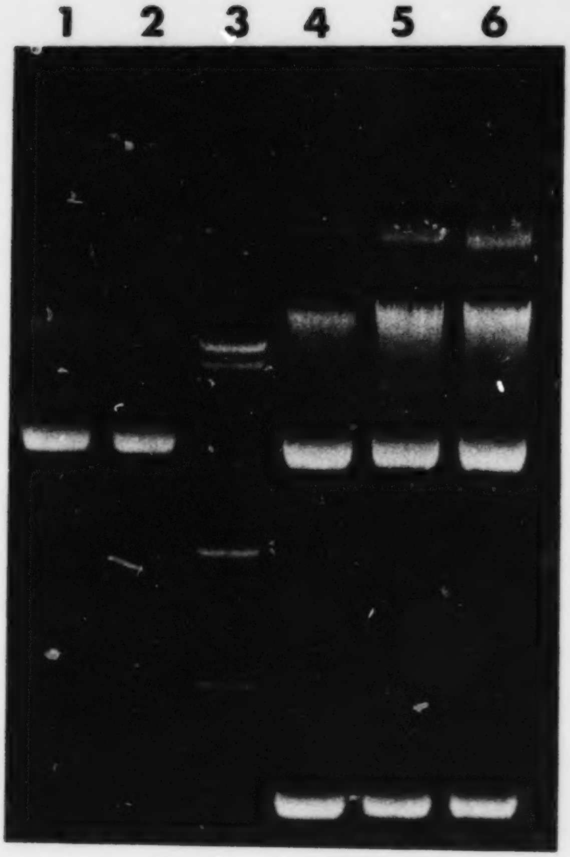

Fig. 2 Plasmid profiles from different strains of *T. ferrooxidans*. Lane 1: TFMSR; Lane 2: TFVS; Lane 3: lambda phage Hind III: Lane 4: TFOS; Lane 5: TFOF; Lane 6: TFOB.

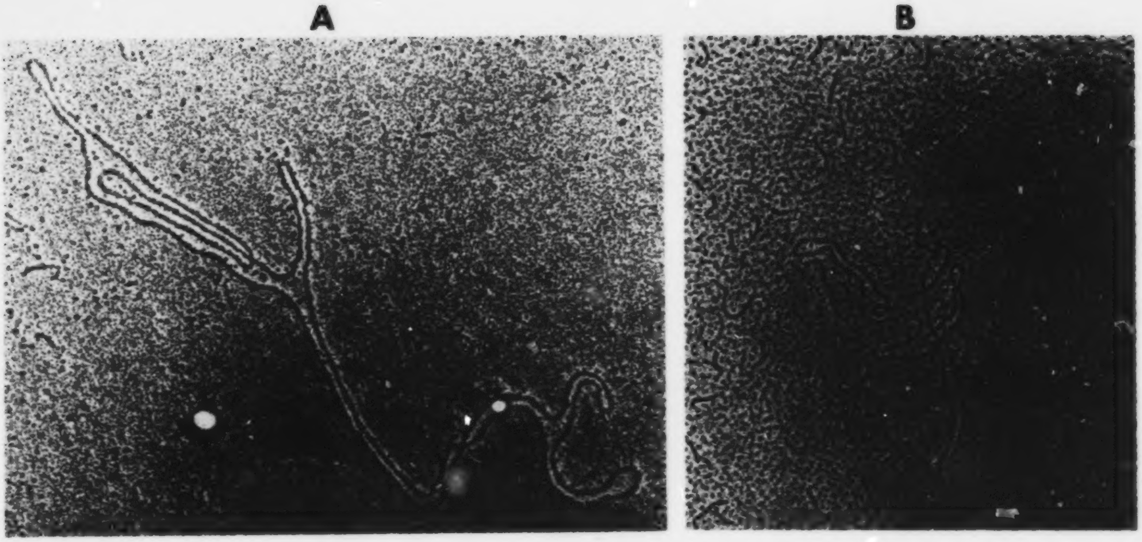

Fig. 3 Electron micrographs of plasmid DNA from *T. ferrooxidans* TFOB. (A) 20 Kb plasmid; (B) 9 Kb plasmid.

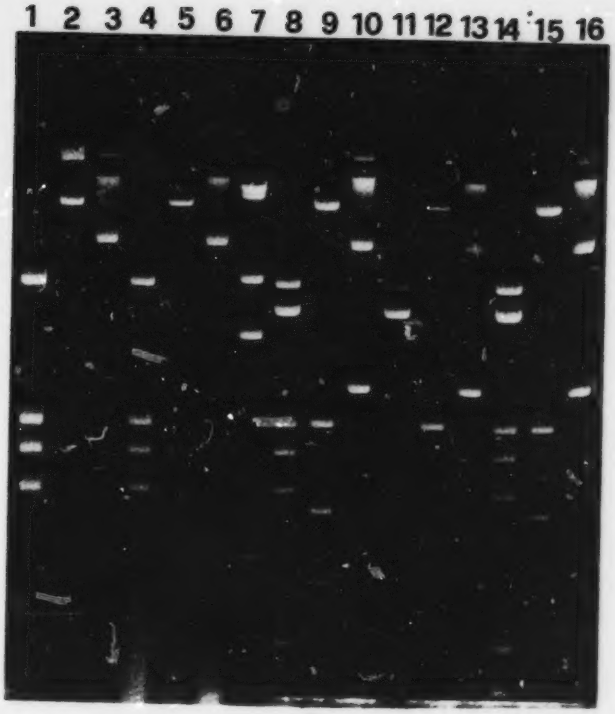
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 4 Plasmid DNA from *T. ferrooxidans* strains TFVS, TFMSR, TFOS, TFOF, TFOB digested respectively with Bam HI (lanes 1,4,8,11,14), Hind III (lanes 2,5,9,12,15) and not digested (lanes 3,6,10,13,16). Lane 7 : Lambda phage Hind III.

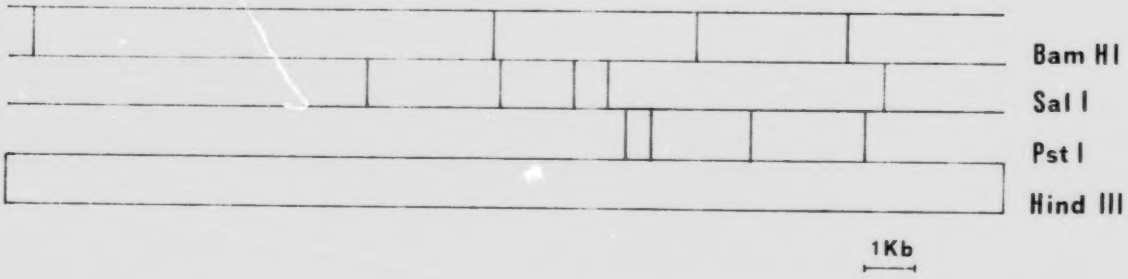
Bam HI
Sal I
Pst I
Hind III
1Kb

Fig. 5 Restriction endonuclease cleavage map of the *T. ferrooxidans* plasmid pTFO.

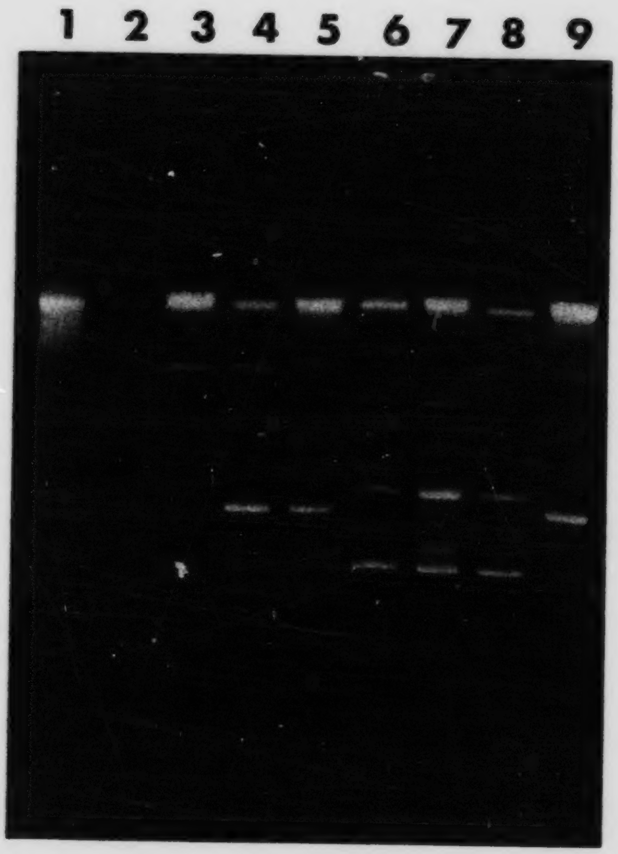

Fig. 6 Plasmid DNA from *T. ferrooxidans* strains TFG02 (lane 1), TFNM3 (lane 2), TFNM1 (lane 3), TFOB (lane 4), TFOF (lane 5), TFMV (lane 6), TFN11 (lane 7), TFFC (lane 8) and TFP4 (lane 9).

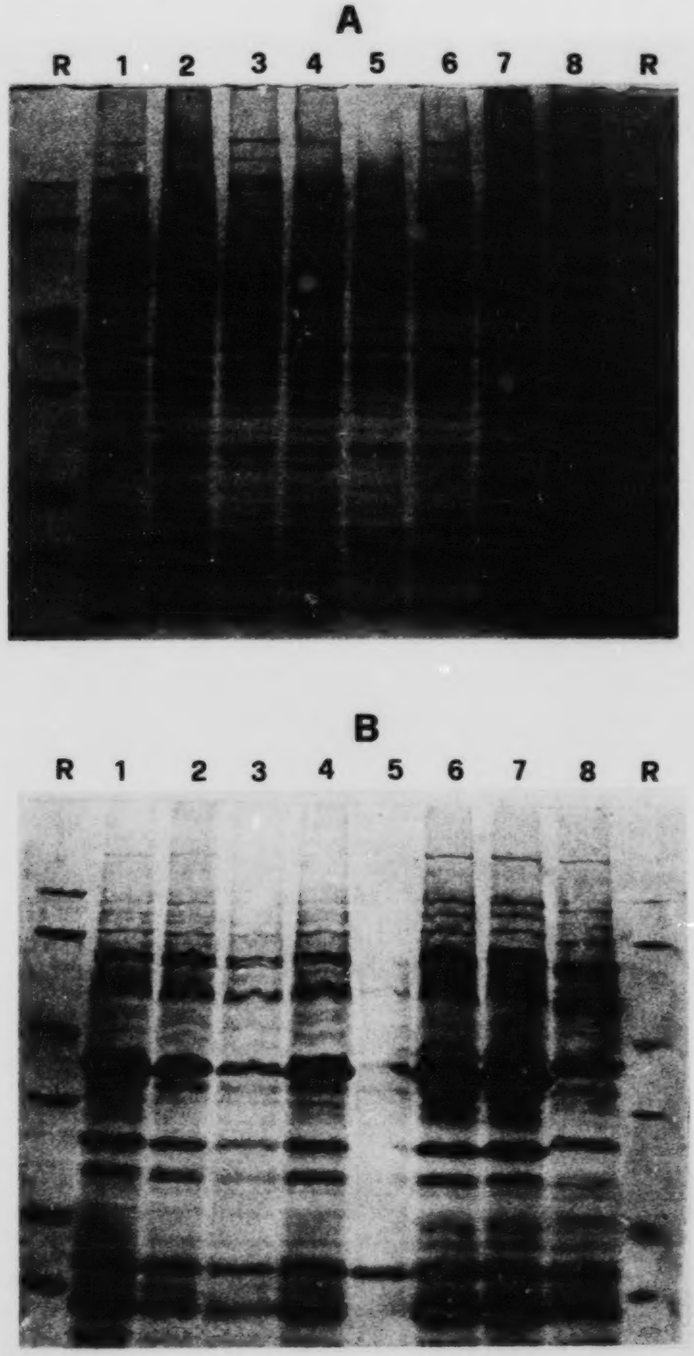

Fig. 7 Polyacrylamide-SDS gel electrophoresis of whole (A) and outer membrane (B) proteins from strains TFOB (lanes 1), TFOF (lanes 2), TFOS (lanes 3), TFVS (lanes 4), TFMSR (lanes 5), TFFC (lanes 6), TFMV (lanes 7) and TFG02 (lanes 8). R : molecular weight markers.

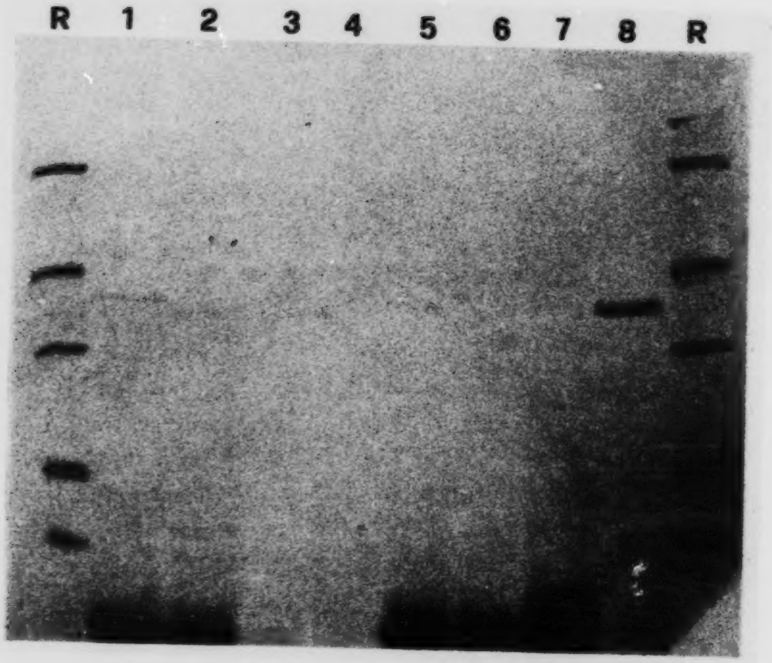

Fig. 8 Polyacrylamide-SDS gel electrophoresis of proteinase K digested whole proteins from strains TFOB (lanes 1), TFOF (lanes 2), TFOS (lanes 3), TFVS (lanes 4), TFMSR (lanes 5), TFFC (lanes 6), TFMV (lanes 7) and TFG02 (lanes 8). R : molecular weight markers.

PRECIOUS METALS

INTEGRATED BIOLOGICAL PROCESSING OF COMPLEX SULPHIDES FOR METALS EXTRACTION AND WASTE TREATMENT

P.B. Marchant, R.W. Lawrence

Coastech Research Inc., 80 Niobe Street, North Vancouver, B.C. V7J 2C9

ABSTRACT

An integrated biological process concept is described comprising two or more microbiologically assisted process steps for the treatment of sulphide ores or concentrates. Such an integrated process allows for the extraction of metal values and for the treatment of the corresponding waste streams for environmental disposal. Metal extraction from base metal ores is achieved through the use of sulphide-oxidizing bacteria such as *T. ferrooxidans*. For refractory sulphidic gold ores, the same bacteria can be used to liberate the precious metal from the sulphide host minerals for recovery by conventional methods such as cyanidation. In either case, a sulphate solution is generated which can be treated in a patented 2-stage anaerobic-aerobic biological process to produce sulphur and reusable water. For the processing of gold ores and concentrates, a further biological process can be effective for the degradation of free cyanide, thiocyanate and metal cyanide complexes in solutions remaining after the gold extraction step. The current state of the art in biological technology is described and potential applications for an integrated approach is discussed.

TRAITEMENT BIOLOGIQUE INTÉGRÉ DE COMPOSÉS SULFUREUX COMPLEXES POUR L'EXTRACTION DE MÉTAUX ET POUR LE TRAITEMENT DE DÉCHETS

P.B. Marchant et R.W. Lawrence

Coastech Research inc.
80 rue Niobe, Vancouver Nord, C.B. V7I 2C9, Canada

RÉSUMÉ

Le concept d'un procédé biologique intégré comportant au moins deux étapes qui font intervenir des microorganismes pour le traitement de minerais ou de concentrés sulfureux est présenté. Un tel procédé permet l'extraction de métaux et du même coup le traitement des eaux polluées que cela occasionne. L'extraction de métaux à partir de minerais de métaux communs est effectuée par des bactéries qui oxydent le sulfure comme *T. ferrooxidans*. Pour des minerais sulfureux réfractaires contenant de l'or, ces bactéries peuvent ensuite être utilisées pour libérer le métal précieux qui ensuite peut être récupérer par des méthodes conventionnelles comme la cyanuration. Dans les deux cas, une solution contenant du sulfate est générée. Elle peut être traitée par un procédé biologique breveté qui comporte deux étapes, anaérobie et aérobie, et qui produit du soufre et de l'eau qui peut être réutilisée. Pour le traitement des minerais et concentrés aurifères, un autre procédé biologique peut être incorporé pour dégrader le cyanure libre, le thiocyanate et les complexes métalliques de cyanure qui restent en solution après l'extraction de l'or. Les techniques biologiques existent actuellement ainsi que les applications possibles pour un système intégré sont présentées.

Mots clés : Composé sulfureux complexe; composé sulfureux réfractaire; affinage des métaux communs; métaux précieux; réduction biologique du sulfate; détoxification biologique du cyanure; soufre élémentaire; traitement des effluents contenant du sulfate; traitement synergique.

KEYWORDS

Complex sulphide; refractory sulphide; base metals refining; precious metals; biological sulphate reduction; biological cyanide detoxification; elemental sulphur; sulphate effluent treatment; synergistic processing.

INTRODUCTION

The mining and milling of sulphidic gold ores is becoming increasingly complex due to the nature of the ores treated, the processes employed for gold extraction, and improved corporate waste management responsibility due to increased environmental legislation and control. Increased interest, research activity, and technical need have improved applications of biotechnological methods in metals extraction and waste treatment to the point that an integrated biological processing flowsheet can be considered. Figure 1 shows, conceptually, the use of biological processing to assist in metals extraction and waste management practice in treating a complex sulphide ore or concentrate. The biologically assisted processing steps include: (i) base metals extraction and refining, (ii) gold extraction and refining by cyanidation, (iii) cyanide waste treatment, and (iv) thiosalt and sulphate by-product treatment.

Briefly, base metal extraction, such as zinc or copper, is achieved in BIO 1, in Figure 1, through the use of sulphide oxidizing bacteria such as *Thiobacillus ferrooxidans*. For refractory sulphidic gold ores and concentrates the same bacteria types can be employed to liberate the precious metal from the sulphidic host, BIO 2, for subsequent gold extraction by cyanidation. The resultant complex cyanide solutions are treated biologically for cyanide detoxification and metals removal, BIO 3, to produce either recycle process water or discharge quality effluent. The final biological treatment step, BIO 4, is incorporated to treat complex acidic sulphate solutions; waste 1A, a thiosalt solution resulting from fine grinding of sulphidic minerals; and waste 1B and 1C, complex sulphate and dissolved metal solutions which are the products of the sulphide oxidative pretreatment steps, BIO 1 and BIO 2. A patented two-stage biological process is employed to reduce sulphate to a final product of elemental sulphur, metals removal to metallo-sulphide waste sludge, a carbonate-rich solution for recycle and pH buffering capacity, and discharge quality water for either process recycle or discharge to the environment.

This paper provides a brief description of the biologically assisted metal extraction processes, which have been reported in detail previously, and a more detailed description of the biological processes involved in cyanide detoxification and sulphate reduction. Finally, an integrated process flowsheet is described that incorporates several biological process steps incorporating both metals extraction and waste treatment.

METALS EXTRACTION

Base Metals

Considerable attention has been directed towards extraction of base metals by bacterial methods. Principally copper, zinc, and uranium have been the focus of process development to date. Biological extraction of copper has been practiced for over 30 years in large copper dump leaching operations (Corrans and co-workers, 1972; Bruynesteyn and Duncan, 1972). Uranium extraction by in-situ sulphide biooxidation is presently practiced by Denison Mines (Gould and McCready, 1989). Typically base metal sulphide concentrates are treated in smelter operation for metal refining. Base metal concentrates are readily bioleached (Lawrence, 1974; Pinches, 1972; Bruynesteyn and Duncan, 1970) and, in some cases, bioleaching might provide an alternative to smelting. Previous data suggests (Lawrence, 1974) that some selective base metal oxidation might be possible as differential rates of biooxidation typically follow, in order: galena, arsenopyrite, sphalerite, chalcopyrite, and pyrite. The resultant sulphate solutions can reach metal concentrations typical of electrowinning operations: 25 g/L Cu and 120 g/L Zn.

Biological treatment of lead concentrates is not as likely as others because the resultant $PbSO_4$ from sulphide biooxidation is insoluble and remains with the residue. Lead extraction using amines is possible. Copper and/or zinc sulphide concentrates are more likely bioleach candidates, especially combined with sulphide recovery for precious metals.

Figure 2 shows a conceptual base metals refining circuit using acidic biooxidation of sulphides to supply lixiviant for base metals. The bioleach product reports to a dewatering/wash step where the sulphate solution is washed from the residue for base metal recovery. The washed residue reports as a final concentrate (for example, after removal of the zinc from a copper-zinc concentrate) or is repulped for further processing (Figure 3). The resultant sulphate solution would report to conventional hydrometallurgical refining processes. Note that waste heat from the biooxidation step can be employed to heat solutions during refining for diluent removal and solution evaporative concentration. The resultant barren sulphate solution can be recycled as required, depending on the acid balance, and/or bled to a sulphate removal step (Figure 4).

Precious Metals

Most sulphides can be oxidized by bacteria (Lawrence, 1974; Pinches, 1972; Malouf, 1970) and consequently highly complex sulphide concentrates containing precious metals can be pretreated for cyanidation by biooxidation. There has been considerable attention focused on bioleaching of refractory gold concentrates recently (Lawrence and Bruynesteyn, 1983; Marchant, 1986; Marchant and Lawrence, 1986) and therefore the details of the process are not described herein.

Figure 3 shows a typical flowsheet incorporating a biooxidation step to enhance gold extraction by cyanidation from a refractory sulphide concentrate. The bioleach process shown here is much the same as that shown in Figure 2. Similarly, the bioleach product reports to a solid-liquid separation step where the sulphate solution is washed from the precious metals residue. The residue is neutralized and cyanide leached by conventional methods. The sulphate solution is either recycled as required and/or bled to a sulphate removal step (Figure 4). Note that the decant solution from the tailing pond, cyanide laden, also reports to the sulphate removal step (Figure 4).

BIOLOGICAL SULPHATE REDUCTION

Sulphate wastes, such as those produced during sulphide biooxidation processes described in Figures 2 and 3, must be neutralized and the metals removed, and can present a serious environmental pollution potential. A biological sulphate removal process has been developed for the treatment of these, and other, sulphate wastes. The process was developed by CSIR (Maree and Strydom, 1985; Maree and Strydom, 1987; Maree and co-workers, 1987a; Maree and co-workers, 1987b) for the reprocessing of gypsum wastes. The process used at Coastech is also applicable to complex sulphate solutions and acid mine wastes typically encountered during sulphide processing.

Briefly, the biological sulphate reduction process operated at Coastech consists of several process stages: anaerobic sulphate reduction, H_2S stripping, aerobic treatment, and sulphur production stages. In the first stage sulphate is reduced to H_2S when an energy source, such as molasses or producer gas, is added. In the second stage, H_2S is stripped from the water. In the third stage, residual organic compounds are biodegraded aerobically and in the last stage H_2S is converted to elemental sulphur. This process is patented in South Africa and the United States, while patent applications in Australia and Canada are pending.

Applications of biological sulphate reduction include:

- stack gas sulphate streams,
- waste gypsum (eg. fertilizer industry during the manufacture of phosphoric acid),

- hydrometallurgical sulphate or ammonium sulphate based extraction process effluents,
- sulphide oxidative pretreatment effluents (eg. bioleaching, roasting, pressure oxidation),
- soluble thiosalt removal following fine grinding of sulphides.
- acid mine drainage

Potential users include operating base metal and/or precious metals mines, copper dump leach operations, roasting operations, smelting operations, metal refineries and plating operations, power stations, and fertilizer manufacturers. The inherent advantages of the sulphate reduction process compared with neutralization with lime and/or limestone include:

(i) elimination or reduction of lime required,

(ii) elimination or reduction of sludge by-products (gypsum and hydroxides), and related scale and ponding capacity,

(iii) a simple carbon source is the only consumable,

(iv) saleable elemental sulphur is the primary co-product,

(v) the final solution product is buffered to pH 7.0 to 7.2 by carbonate by-product,

(vi) simple process control and process flexibility.

The sulphate reduction process is described in more detail in Figure 4. Each unit process can be described:

Anaerobic Stage

In the anaerobic stage, molasses is fermented to lactic acid, which is used as carbon source by the sulphate reducing bacteria to produce H_2S and fatty acids such as acetic acid. Other carbon sources can be employed, such as producer gas, resulting in a simpler overall reaction, and a carrier gas source. Toxic heavy metals in the water are precipitated as the corresponding metal sulphides. The fact that alkalinity is produced, makes the direct neutralization of acidic water possible. Traditionally, acid water, such as acid mine drainage, is neutralized with lime. With the sulphate reduction process, both neutralization and sulphate reduction can be achieved after addition of a carbon source. In the case of alkaline sulphate solutions a slight pH rise might be expected. The reactions that take place in this stage are the following:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4CH_3CHOHCOOH \qquad (1)$$

$$2CH_3CHOHCOO^- + SO_4{=} \rightarrow 2CH_3COO^- + 2HCO_2^- + H_2S \qquad (2)$$

(sulphate reduction and alkalinity production)

$$M^{2+} + S^{=} \rightarrow MS \qquad (3)$$

(heavy metal precipitation)

Where a simpler carbon source is employed the net reaction can be represented by:

$$2C + SO_4^{=} + 2H_2O \rightarrow H_2S + 2HCO_3^- \qquad (4)$$

Stripping Stage

The residual H_2S in solution after the anaerobic stage, is stripped with a carrier gas. The mixture is then transported to the sulphur production stage.

$$H_2S(aq) + \text{carrier gas} \rightarrow H_2S(g) + \text{carrier gas} \qquad (5)$$

(mass transfer from liquid to gas phase)

Aerobic Stage

The effluent from the stripping stage contains a high co10centration of organics which need to be biodegraded in the aerobic stage. Here the metabolic end-products from sulphate reduction, such as acetic acid, are converted to CO_2 and the residual sulphide oxidized to sulphate. The net result so far is that sulphate has been replaced by carbonate. This causes partial desalination of the water. Cyanide, if present, is also biodegraded at this stage. These reactions could be represented as follows:

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O \qquad (6)$$

(aerobic biodegradation)

$$S^{=} + 2O_2 \rightarrow SO_4^{=} \qquad (7)$$

(su0lphide oxidation)

Note that $CaCO_3$ is produced when sufficient calcium occurs in the feed, otherwise a saturated HCO_3^- product pr1evails (see reaction (4)).

$$Ca^{2+} + CO_3^{=} \rightarrow CaCO_3 \qquad (8)$$

Sulphur Production Stage

In the sulphur production stage, hydrogen sulphide is oxidized to elemental sulphur by bubbling it through an iron (III) solution.

$$H_2S + 2Fe^{3+} \rightarrow S^{o} + 2Fe^{2+} + 2H^{+} \qquad (9)$$

(sulphur production)

Where the Fe^{3+} can be regenerated internally using Ferrobacillus ferrooxidans and air, or the sulphur production can be accomplished photosynthetically as shown in Figure 4:

$$2H_2S + CO_2 \xrightarrow{\text{light}} 2S^{o} + C + 2H_2O \qquad (10)$$

Secondary Anaerobic Stage

A second anaerobic stage is incorporated in Figure 4 to ensure reaction completion, especially if a complex carbon source is employed.

The final solution, HCO_3^- rich, can be recycled as required internally to the sulphate reduction process and as a neutralization source elsewhere during sulphide processing and as a source of CO_2 during sulphide biooxidation. Note that the carrier gas for H_2S stripping (N_2) can be recycled. If producer gas is used (CO, CO_2, N_2) all of the necessary raw materials are supplied to the anaerobic stage as a single gaseous source.

Additional detail on the biological sulphate reduction process has been described (Marchant and co-workers, 1989).

BIOLOGICAL CYANIDE DETOXIFICATION

Biological cyanide detoxification processes take advantage of naturally occurring biochemical mechanisms to convert and/or metabolize cyanide species to inert compounds and fix heavy metals derived from the decomposition of soluble complex cyanide species. This section will briefly describe the nature of the biomass employed in cyanide detoxification, the primary mechanisms involved, processing alternatives, some recent results from commercial scale operations of Homestake Mining and Gold Fields Mining employing biological cyanide treatment, and application of the sulphate reduction process shown in Figure 4 for cyanide and complex cyanide detoxification.

Biomass and Mechanisms

Biomass used in cyanide detoxification include, but are not limited to:

Fungi — Fusarium, Pholiota, Rhizopus
  Bacteria — Bacilli, Thiobacilli, Actinomyces, Pseudomonas

Successful cyanide treatment systems are generally heterogeneous populations, where co-metabolism is the important overall mechanism. In other words, the sequential breakdown of cyanide compounds by a number of different micro-organisms. This characterization of mixed cultures has interesting practical applications for selective use of micro-organisms and process objective. Most cyanide tolerant micro-organisms are naturally occurring in various soils.

There are no general mechanisms of cyanide detoxification by biological routes due to the wide range of operating conditions, biomass, cyanide species, and solution chemistry. The mechanisms can, however, be classified into three primary groups:

Metabolism
Adsorption
Stripping/absorption

The long term objective is biological degradation of cyanide to inert constituents by metabolic processes. The metabolic processes can be divided into two groups:

Assimilation
Conversion

where assimilation describes complete degradation of the cyanide as a carbon and/or nitrogen source in metabolic pathways ultimately as amino acid or protein compounds within the cellular milieux. Usually a secondary source of carbon, such as glucose or other easily oxidized hydrocarbon, and nutrients, such as phosphate, are required to enhance the rate of cellular growth and thus cyanide degradation. Cyanide typically interferes with cellular oxidation processes, by binding with the iron group of cytochrome oxidase, and therefore inhibiting oxygen use for cellular metabolism. Those organisms that have developed a cyanide resistant cytochrome oxidase path have the ability to assimilate cyanide species.

Conversion usually describes the use of cyanide as a precursor in some metabolic pathway, assisted by various enzymes such as formamide hydrolase, cyanide hydratase, rhodanese, and various other synthase compounds. There is no assimilation of either carbon or nitrogen. Typical by-products of cyanide degradation include carbon dioxide and ammonia.

Adsorption and stripping are generally the first mechanisms of cyanide removal from solution and are attributed to cell wall interactions such as polysaccharide matrices of flocculating bacteria. Stripping and adsorption are common mechanisms from solutions >50 mg/L CNT, however, the ultimate fate of cyanide is some form of metabolism as there is generally no accumulation of cyanide species in the biological sludge. The rate of metabolism will vary from one system to another depending on a number of variables such as substrate, mass transfer, solution chemistry, temperature, nutrients, pH, SCN/CN ratio.

Spore forming micro-organisms are particularly important due to the resistance of spores to mechanical and chemical factors. The relative toxicity of metals in solution varies greatly depending on the biomass employed and prevailing operating conditions. Generally, metallo-cyanide complexes are biodegradable in much the same fashion as free cyanide species.

Thiocyanate is degraded rapidly by either direct assimilation of the carbon and nitrogen and an oxidized sulphur compound or by conversion to cyanate and elemental sulphur and subsequent conversion to CO_2, ammonia, and sulphate.

Processing Alternatives

Commonly practiced processing mechanisms for biological cyanide degradation are shown in Table 1. The choice will depend on the type of solution for treatment, the nature of the operations (eg. continuous, large scale, etc.), and the ultimate route for the treated product. In general, bleed solutions are the most easily treated, especially if there is a dilution source prior to biological degradation. If the objective is to treat a heap leach solution it is best to treat the pond rather than the heap, for control purposes. If a slurry effluent is treated, more sophisticated reactors must be employed.

TABLE 1

Processing Equipment Alternatives

SOLUTIONS	SLURRIES
Trickling Filters	Splash Towers
Activated Sludge	CSTR
Extended Aeration	
Fixed Bed Enzyme Columns	
Fluidized Bed Reactors	
Fixed Film Reactors	
Rotating Biological Contactors (RBC)	
Splash Towers	
Aerated Filters	
CSTR	

For solutions, the most common processing equipment anticipated on a large scale include fixed film and rotating biological contactors, aerated filters, and stirred tank reactors (CSTR). For slurries, to improve substrate availability and degradation kinetics, equipment will be limited to splash towers and CSTR's. For integrated biological processing the equipment used for sulphate reduction, Figure 4, will be common to both sulphate solution processing and cyanide detoxification. The process is not applicable to cyanide laden slurries.

Applications and Results

The first commercial operation in a mining application treating cyanide laden milling effluent streams biologically was reported by Homestake Mining Corporation at their Lead, South Dakota operations. A summary of published results is shown in Table 2, from commercial scale rotating biological contactor operations treating a diluted cyanide contaminated bleed stream using Homestakes' patented biological cyanide treatment process:

TABLE 2

Homestake RBC Operating Results (Whitlock and Mudder, 1986)

	(mg/L)	
Solution Constituent	Feed	Product
CNS	45 - 75	<0.1
CN	1 - 10	0.3
CNT	0.5 - 5.0	0.02
Fe	0.5 - 2.0	<0.1
Cu	0.1 - 1.5	0.05
Ammonia	1.0 - 6.0	0.10

Gold Fields Mining Corporation has reported data (Thompson, 1989) from operating heap leach operations where total cyanide levels in outflow solutions degraded form 150 mg/L to 30 mg/L after only six months of weathering. However, total cyanide levels of 10-30 mg/L persisted after four years. The importance of biological enhanced heap detoxification is apparent from these data.

Gold Fields trials using mixed biological cultures showed:

TABLE 3

Gold Fields Test Data

		(mg/L) CNT	
	Bottle	Column	Field
Test	123 to <0.1	125 to <0.1	77 to 3.8
Control	117 to 53	127 to 49	27 to 23

The process shown in Figure 4 can be employed for cyanide detoxification. Metallo-cyanide species are reduced in the anaerobic reactor to the corresponding metallo-sulphide, when sufficient sulphate is present, and free cyanide and/or HCN. The free cyanide and HCN (stabilized in solution by HCO_3^-) report to the aerobic stage where the cyanide is decomposed by various pathways described above. Note that a solution mixing step should be incorporated where sulphate and cyanide solutions are combined, along with HCO_3^- rich recycle solutions. Results to date indicate <1 mg/L CNT in the treated solution following aerobic processing and metals effectively removed.

Comparison with Alternatives

Comparing biological cyanide degradation with chemical processes shows:

TABLE 4

Comparison of Alternatives

Process	CNS	CNT	Ammonia	Metals
Alkaline Chlorination	Yes	No	Yes	No
SO_2/air (Cu)	No	Yes	No	Yes
Peroxide (Cu)	No	Yes	No	Yes
A/V/R	No	Yes	No	Yes
Biological	Yes	Yes	Yes	Yes

The biological processing alternative is apparently competitive with alternatives technically. Note that there is no benefit of cyanide recovery such as that with A/V/R technology. An important long term benefit of biological processing is the complete removal of thiocyanate species.

Limitations

Biological cyanide degradation is presently limited to relatively dilute solutions of simple solution chemistry and relatively high SCN/CN ratios. Improved toxicity tolerance is a function of biomass acclimation and can be accelerated by standard microbiological methods.

INTEGRATED PROCESSING

Figure 5 describes the integration of the unit processes shown in Figures 2, 3, and 4. The synergistic process proposed incorporates base metal bioleach extraction from a complex sulphide concentrate followed by secondary biooxidation to enhance precious metals recovery by cyanidation. The combined sulphate waste solutions are combined with cyanide laden tailings decant solution and base metal solution refining reject precipitates and treated in a biological sulphate reduction step to produce recycle process water and water for discharge to the environment. The final commercial products include a base metal electrowinning product, elemental sulphur, precious metals bullion, and reusable water. There is potential for secondary metal production by refining the sulphide sludge from the anaerobic reactor during sulphate reduction, depending on the feed type and grade.

Process synergism is demonstrated in the following areas:

- combined treatment of sulphate solutions produced during biooxidation and metallic precipitates from base metal solution refining in a common biological sulphate process producing saleable elemental sulphur from sulphate waste and a more stable metal sulphide solids product,
- CO_2 generated during aerobic processing and secondary biooxidation residue neutralization can be used as a carbon source for both biooxidation stages,
- final sulphate reduction solution, carbonate-rich, can be recycled for use as a pH control during biooxidation, bioleach residue wash water, and as a neutralizing source prior to cyanidation,
- the sulphate reduction process can co-treat complex metallo-cyanide solutions to remove soluble metals and decompose cyanide species, which allows cyanide process water recycle to bioleach processing,
- reuse of cooling water from bioleach processing to heat base metal solutions for refining purposes and evaporative concentration of both base metal refining solutions and carbonate recycle water. Integrated cooling and heating would be especially attractive if the refractory sulphide biooxidation step is conducted at elevated temperatures (Lawrence and Marchant, 1987; Brierley and Brierley, 1986).

It is apparent that biological processing has a wide range of application in sulphide processing. It has been shown here that biological processes can be integrated to assist in the extraction and refining of both base and precious metals from sulphide minerals and concurrently produce saleable co-products, eg. elemental sulphur, reusable or discharge quality water, and terminal destruction or removal of potential environmental contaminants. There is an increasing understanding of mixed culture systems and potential synergistic processes using biological techniques that are both versatile and resilient. All of the biological processes described herein are operational at Coastech. However, due to the complexity of the processes and feed materials involved, each case must be tested, demonstrated, examined for economic viability, and sound engineering principles applied for confident commercial evaluation of integrated biological processing as proposed.

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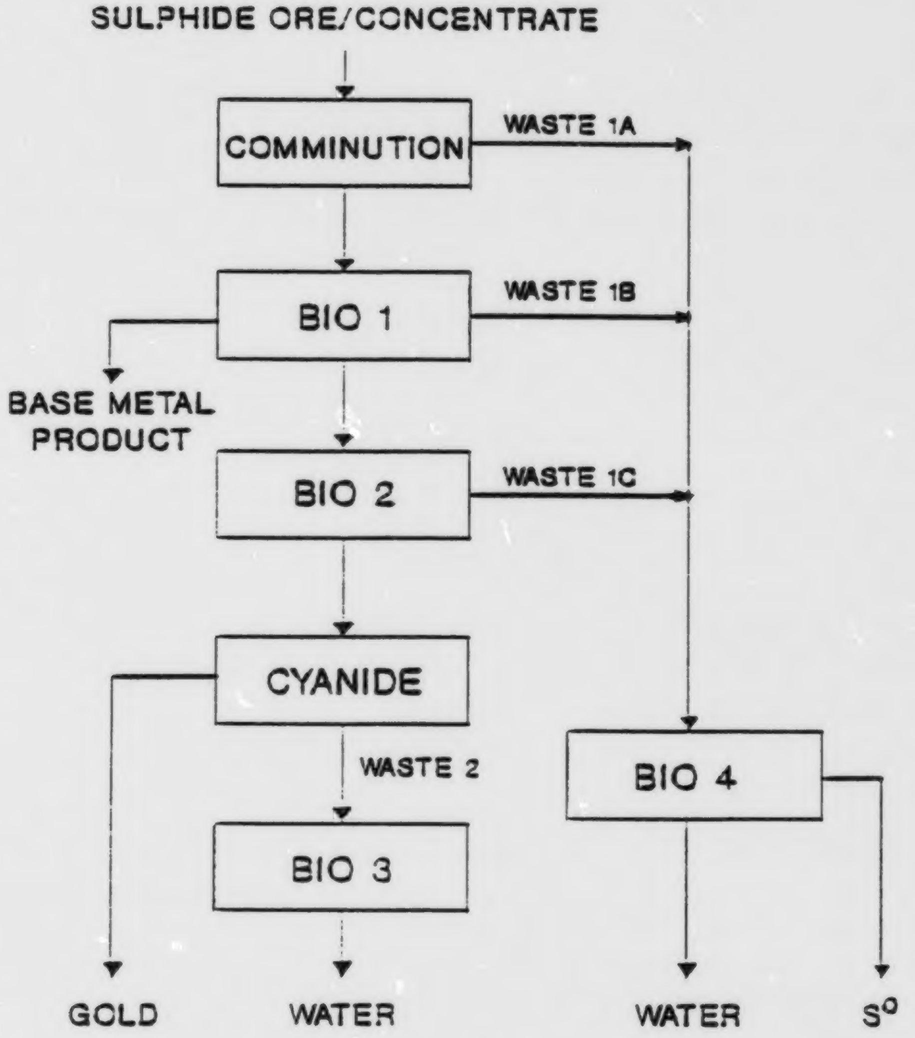
SULPHIDE ORE/CONCENTRATE
COMMINUTION
WASTE 1A
BIO 1
WASTE 1B
BASE METAL PRODUCT
BIO 2
WASTE 1C
CYANIDE
WASTE 2
BIO 3
BIO 4
GOLD
WATER
WATER
S°

Fig. 1 Conceptual integrated biological processing

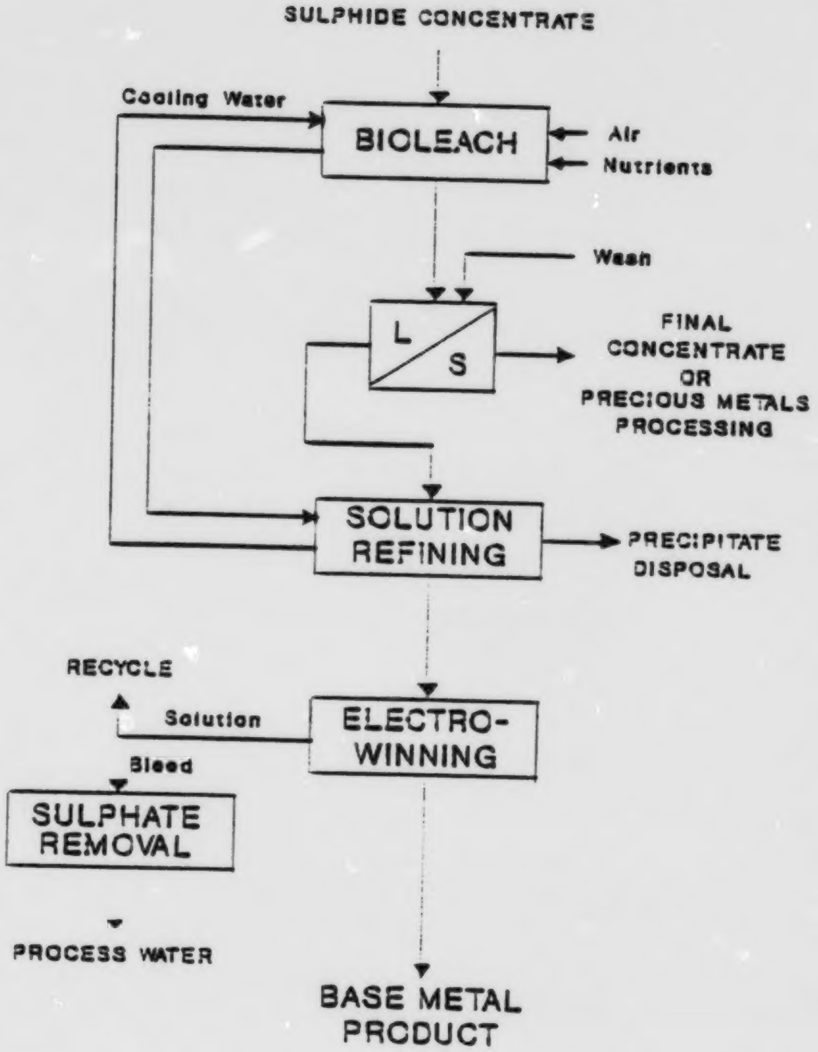
SULPHIDE CONCENTRATE
Cooling Water
BIOLEACH
Air
Nutrients
Wash
L
S
FINAL CONCENTRATE OR PRECIOUS METALS PROCESSING
SOLUTION REFINING
PRECIPITATE DISPOSAL
RECYCLE
Solution
Bleed
ELECTRO-WINNING
SULPHATE REMOVAL
PROCESS WATER
BASE METAL PRODUCT

Fig. 2 Base metal concentrate refining

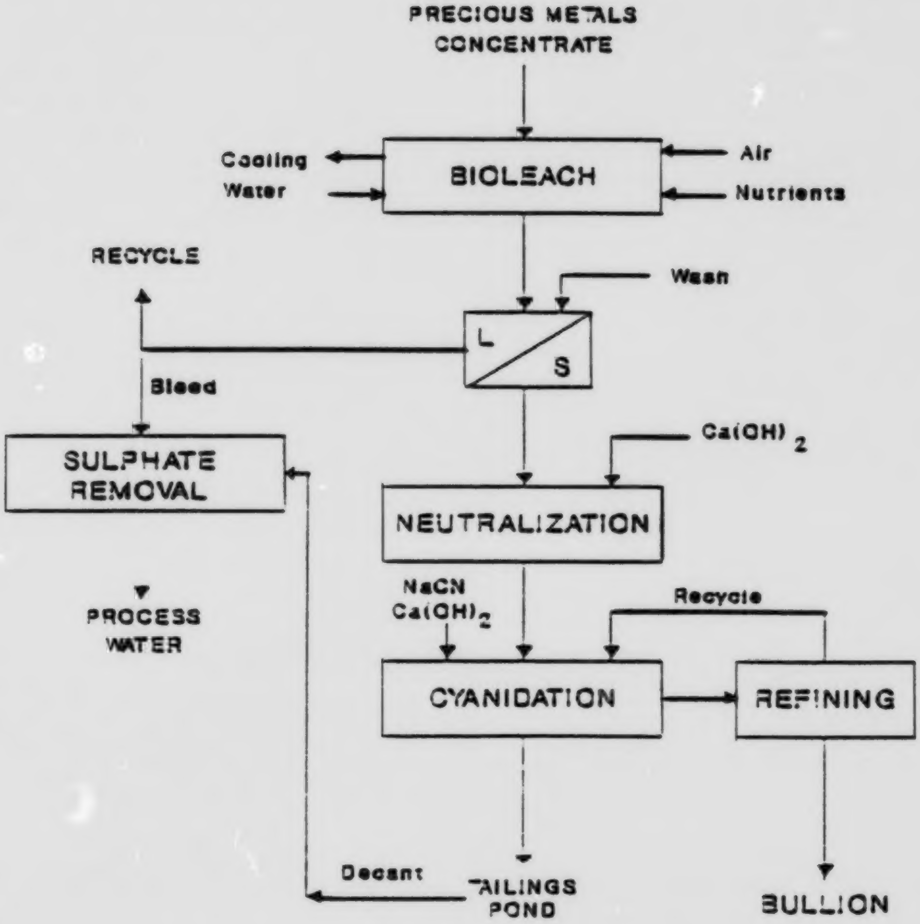
PRECIOUS METALS CONCENTRATE
Cooling Water
BIOLEACH
Air
Nutrients
RECYCLE
Wash
L
S
Bleed
SULPHATE REMOVAL
Ca(OH)2
NEUTRALIZATION
PROCESS WATER
NaCN
Ca(OH)2
Recycle
CYANIDATION
REFINING
Decant
TAILINGS POND
BULLION

Fig. 3 Precious metal concentrate bioleaching

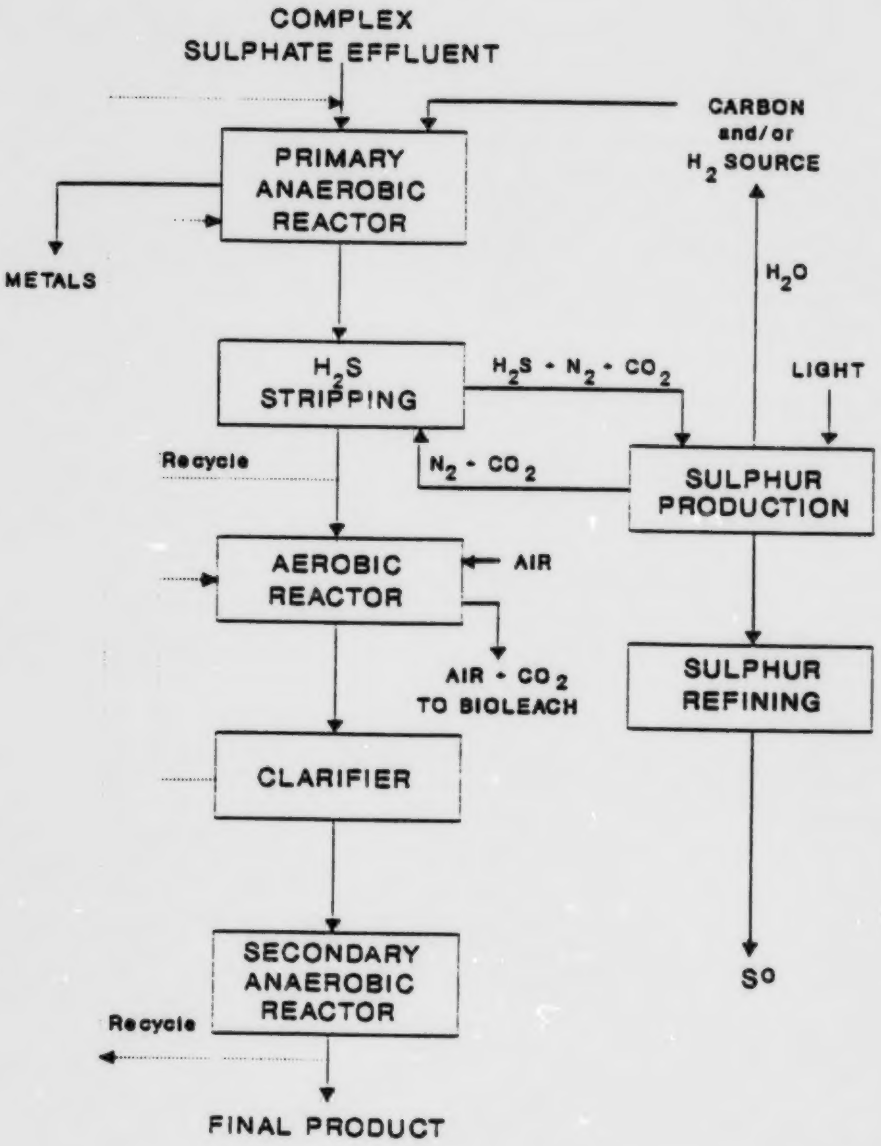
COMPLEX SULPHATE EFFLUENT
CARBON and/or H_2 SOURCE
PRIMARY ANAEROBIC REACTOR
METALS
H_2O
H_2S STRIPPING
$H_2S + N_2 + CO_2$
LIGHT
Recycle
$N_2 + CO_2$
SULPHUR PRODUCTION
AEROBIC REACTOR
AIR
AIR + CO_2 TO BIOLEACH
SULPHUR REFINING
CLARIFIER
SECONDARY ANAEROBIC REACTOR
Recycle
S^0
FINAL PRODUCT

Fig. 4 Complex sulphate solution reduction and elemental sulphur production

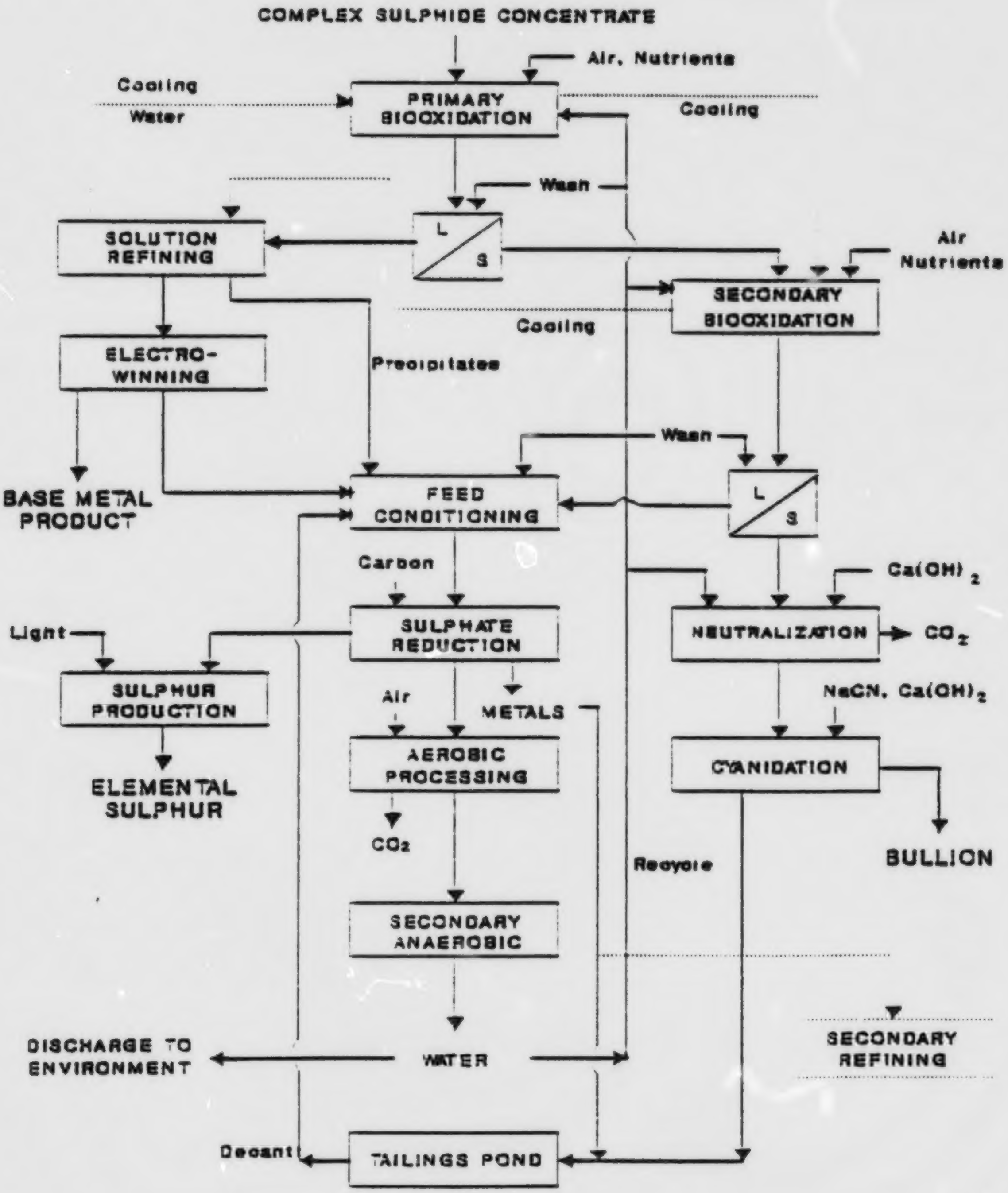
COMPLEX SULPHIDE CONCENTRATE
Air, Nutrients
Cooling Water
PRIMARY BIOOXIDATION
Cooling
Wash
L
S
SOLUTION REFINING
Air Nutrients
SECONDARY BIOOXIDATION
Cooling
ELECTRO-WINNING
Precipitates
Wash
BASE METAL PRODUCT
FEED CONDITIONING
L
S
Carbon
Ca(OH)2
Light
SULPHATE REDUCTION
NEUTRALIZATION
CO2
SULPHUR PRODUCTION
Air
METALS
NaCN, Ca(OH)2
AEROBIC PROCESSING
CYANIDATION
ELEMENTAL SULPHUR
CO2
Recycle
BULLION
SECONDARY ANAEROBIC
SECONDARY REFINING
DISCHARGE TO ENVIRONMENT
WATER
Decant
TAILINGS POND

Fig. 5 Integrated biological processing of a complex sulphide concentrate

BIOREACTOR SCALE-UP: PRACTICAL CONSIDERATIONS FOR BIOLOGICALLY ASSISTED GOLD RECOVERY

*E. A. Griffin * and L. Luinstra*

Eimco Process Equipment 414 West 300 South Salt Lake City, Utah 84110

ABSTRACT

A bioreactor must be designed to meet the specific needs and constraints of a particular process in order to achieve production goals. Scale-up of bioreactor prototypes involves progressing from a laboratory-scale investigation to a practical and economical industrial level. As portions of the mining/mineral industry contemplate installing large-scale continuous bioreactor systems for biologically assisted metal recovery, many issues must be considered.

This paper discusses a practical approach to bioreactor scale-up that was developed by EIMCO's bioleach research team as they focused on improved engineering technology and reaction kinetics.

MISE AU POINT D'UN BIORÉACTEUR DESTINÉ À L'INDUSTRIE : CONSIDÉRATIONS PRATIQUES DANS LE CAS DE LA RÉCUPÉRATION DE PARTICULES D'OR PAR UN PROCÉDÉ BACTÉRIEN.

*E. A. Griffin * et L. Luinstra*

Eimco Process Equipment
414 ouest 300 sud Salt Lake City, Utah É.-U.

RÉSUMÉ

Pour que les objectifs de production puissent être atteints, un bioréacteur doit être conçu de façon à remplir les besoins spécifiques et à satisfaire aux contraintes d'un procédé donné. Pour qu'un bioréacteur soit utilisable dans l'industrie, il faut augmenter la taille du prototype de laboratoire. Comme certaines industries oeuvrant dans les domaines minier et métallurgique comptent installer des bioréacteurs de taille industrielle pour la récupération des particules de métal par procédé bactérien, il faut prendre de nombreux facteurs en considération.

Cet article traite de l'approche pratique développée par l'équipe de recherche en biolixiviation d'EIMCO pour augmenter la taille des bioréacteurs. L'équipe s'est concentrée sur l'amélioration technique des réacteurs et sur la cinétique des réactions.

INTRODUCTION

Although biologically-assisted heap leaching has been practiced for many years, the direct bacterial leaching of sulfide minerals for the purpose of improving gold extraction from refractory ores (bioleaching) has recently come under consideration by the mineral processing industry. The use of bacteria to enhance gold recoveries has been adequately demonstrated at the laboratory and pilot plant level (1, 2, 3). Process scale-up has been complicated by the lack of full-scale bioleaching equipment.

Scale-up is not a simple matter of multiplication. The parameters that govern growth of microorganisms in shaker flasks are not necessarily the same ones that apply at the hundred, thousand, or several hundred thousand liter level. Bioleaching process kinetics are complicated by the interaction between microbial growth and rate of mineral solubilization.

Commercial feasibility of the bioleaching process depends upon the ability of the full-scale system to meet design goals as defined during laboratory and pilot test work. Full-scale bioleaching circuits must be able to support an active biomass with a high cell density (10^6 - 10^9 bacteria/ml) and minimize mass transfer limitations with respect to oxygen, carbon dioxide, and other nutrients. In order to achieve maximum kinetics, a continuous process with balanced flow rates and the ability to control buildup of inhibitory components over time is essential.

Practical aspects addressed by EIMCO during the development of an innovative bioleaching bioreactor are discussed in the following sections.

THE EIMCO BIOLEACH PROJECT

EIMCO has been involved in research dedicated toward the development of a bioreactor capable of handling the bio-oxidation of refractory gold and silver ores and concentrates for over five years. During this time, over twenty different refractory samples have undergone bio-oxidation in the EIMCO equipment.

Testing a variety of materials has allowed evaluation of equipment performance under a wide range of operating conditions. Processing feeds with a sulfur content ranging from 3 - 40%, arsenic levels up to 12%, and oxygen demand up to 1,500 mg O_2/liter slurry/hour resulted in a number of modifications to the bioreactor and defined design criteria with respect to operating variables.

The EIMCO Bioleach process utilizes naturally occurring microorganisms that oxidize pyrite and arsenopyrite as part of normal metabolism. Bacterial leaching refers to the role of bacteria, primarily *Thiobacillus ferrooxidans*, but also *Leptospirrillum ferrooxidans*, and other species of *Thiobacillus* in the solubilization of minerals. These bacteria thrive in an aerobic, acidic, inorganic environment, and derive energy from the oxidation of reduced inorganic sulfur compounds and ferrous iron. During bacterial activity, the surface characteristics of the sulfide mineral particles are changed resulting in improved accessibility to the gold by cyanide solutions during subsequent processing.

To accommodate the bacterial process, the bioreactor has been designed to maintain a healthy and prolific culture of bacteria and to allow control of the process environment. The EIMCO bioreactor is basically a modified slurry agitator. A thickener drive is used to rotate rake arms that support the diffuser-based aeration system. The reactor is constructed of materials that can withstand the strong oxidizing environment and that are not toxic to the micro-organisms. The reactor is illustrated in Figure 1.

Fine bubble aeration is accomplished by passing air through flexible membrane diffusers mounted on the rotating rake arms. This also provides considerable turbulence for mixing purposes. The diffuser material is selected to withstand the abrasive nature of the biooxidation slurry. Solids that settle on the bottom of the tank are raked to the center where an airlift pumps them back to the top for resuspension.

The tank is baffled to enhance mixing, and when increased turbulence is required, an impeller is mounted on the airlift shaft to provide additional mixing. In that situation, the aeration system is separated from mechanical mixing. The reactor can be equipped with a variable speed drive, and, depending on the application, any number of reactors are arranged in a cascading system to permit continuous feed and overflow.

REACTION KINETICS

The primary scale-up factor for a given bioleach system focuses on the reaction kinetics. For bioreactor development and scale-up purposes, EIMCO used experimental data from laboratory and pilot bioleaching tests to provide an estimation of rate controlling steps.

Pilot plants which incorporate EIMCO 60-liter bioleach reactors are assembled in cascading fashion to permit continuous overflow to the succeeding stage. Three stages are normally required for a bioleaching circuit. Bacterial growth is initiated in the first stage, often it is prudent to split the first stage between two reactors to allow greater residence time. The second stage generally exhibits the highest kinetic rates, as bacterial attachment has already occurred and ample sulfide is still available for consumption. The third stage normally exhibits the lowest kinetic rates since the food supply is essentially exhausted. This final stage operates at nearly constant sulfide concentrations, which by necessity is the design sulfide content necessary to achieve the desired gold extraction.

The EIMCO bioslurry reactor behaves as a continuous back- mixed reactor, i.e., each mineral particle has an equal probability of exiting through the overflow at any given time. Since the reactor operates at steady-state, the kinetics are not time dependent and the reaction rate can mathematically be set equal to the difference between mass quantities of oxidized aqueous products exiting and entering the reactor per unit time, divided by reactor volume. Measuring the kinetic rate involves two steps: 1) measuring liquid flows into and out of the reactor; and, 2) analyzing influent and effluent liquid flows for dissolved iron, arsenic, and sulfate. Typical kinetic data for a bioleach circuit operating on an arsenopyrite concentrate are given in Table 1.

PRACTICAL CONSIDERATIONS DURING BIOREACTOR SCALE-UP

Before bioreactor scale-up occurs, the basic bioleaching operating parameters for the particular ore or concentrate of interest must have been examined. The following lists parameters and specific considerations:

- Sulfide content: Expected process ranges and the ability of the culture to adapt to variations in the feed material should be assessed.
- Sulfide oxidation: The degree of oxidation required to achieve the desired gold recovery must be determined.
- Temperature: To achieve optimum productivity, the temperature of the bioreactor must be maintained within a fairly narrow range (32-42°C). The sensitivity of the culture to swings in temperature should be examined, and methods of heat control evaluated.
- Pulp density: Careful consideration should be given to the percent solids as this parameter impacts residence time, bacterial activity, oxygen transfer, and mechanical handling.
- Feed particle size distribution: The optimum particle size distribution is dependent upon the particular feed material. To establish the optimum distribution, a range of particle size distributions and varying degrees of biooxidation should be investigated. The cost of grinding the feed material and the improvement in gold recoveries due strictly to regrind must be considered.
- Dissolved oxygen: The information available concerning background dissolved oxygen content is

contradictory (4,5) and should be determined by laboratory test work.

- Bacterial Strain Adaptation: It is important to use a culture that has been well adapted.
- Nutrient addition: In addition to O_2 and CO_2, it may be necessary to add trace nutrients not present in the feed material (Mg, K, N' PO_4).
- pH: In order to avoid jarosite formation and culture shifts, the pH should range between 1-2. Strategies for maintaining pH control within the reactors should be determined during pilot test work.
- Bacterial inhibitors: Inhibitors and toxins must be identified. Care should be taken to avoid using materials of construction that may repress the culture.

Efficient oxygen transfer is a dominant design feature for a bioleach reactor. Overall process economics are influenced by power cost incurred when meeting extremely high oxygen demands. Solids suspension is another important design feature, especially in concentrate oxidation, since the solid particles have high specific gravities, and if allowed to settle, will pack tightly. Resuspension of coarse materials is required to avoid accumulation of solids within the bioreactor. The need for localized turbulence has been identified as a factor in providing an adequate oxygen supply to the bacteria and as an aid in removal of accumulated by-products from particle surfaces. Oxygen transfer and mixing considerations are discussed in more detail in the following sections.

OXYGEN TRANSFER

Though it has been reported that *T. ferrooxidans* is capable of oxidizing sulfur anaerobically (6), the chemistry of primary interest involves oxidation under aerobic conditions, therefore, oxygen is an essential feed stream. Oxygen transfer requirements must be carefully considered during design as the bioreactor environment must achieve an acceptable oxygen mass transfer capability at a minimum cost. If the oxygen transfer ability of the equipment is exceeded by the bacteria's ability to consume oxygen, the soluble oxygen will be depleted by the bacteria. As bacterial kinetics drop due to lack of oxygen, the soluble oxygen is restored, and in time, the bacterial culture will adapt to the oxygen limitation. System cycling will occur and this will result in slower kinetics since the process is operating well below its potential.

Design of the full-scale system requires detailed oxygen demand data for the culture involved. Rates of oxygen consumption in a continuous process will vary between reactor stages as indicated by Table 2. Oxygen uptakes exceeding 1,500 mg O_2/liter slurry/hour have been encountered during pilot plant test work in the EIMCO Bioleach lab.

In order to minimize aeration costs, the actual oxygen demand should be determined and the minimum air flow rates for adequate oxygen supply to the bacterial population should be defined.

Techniques have been established to measure how well an existing bioreactor system is transferring oxygen. The measurement of the mass transfer coefficient for oxygen (K_La) is a useful way to characterize bioreactors for aerobic processes. The K_L term is the liquid-film coefficient for the transfer of oxygen from the air bubble into the slurry and is usually the rate controlling term for oxygen transfer. The interfacial area per unit volume is represented by a and is grouped with the transfer coefficient because of the difficulty to determine this area.

The oxygen mass transfer rate equation is shown in Figure 2. Under steady state conditions, dc/dt is zero, and the rate of oxygen utilization should equal the rate of oxygen dissolution. Alpha and beta are used to extrapolate clean water data to oxygen transfer in a bioleach slurry. When alpha is greater than 1.0 and beta is less than 1.0 (typical of a bioleach system), the background concentration, C, has a definite effect on oxygen transfer. Background dissolved oxygen concentrations of at least 2 mg/liter slurry are maintained in the EIMCO bioslurry reactor to ensure that dissolved oxygen availability is not limiting the process (5). The effect of static head at the bottom of the reactor and calculation of an equilibrium oxygen concentration that represents an average of top and bottom values are needed when evaluating tanks larger than 500 liters.

Aeration rate is defined as the absolute amount of oxygen available to transfer into the system. Oxygen transfer in the EIMCO bioreactor is accomplished by passing air through fine bubble diffusers mounted on the rake arms which rotate near the bottom of the tank. The process air enters the bioreactor through a rotary valve above the reactor drive, proceeds down through the center column, and is distributed to the diffuser manifolds (see Figure 1). This design promotes decreased bubble coalescence, reduction of the average bubble size diameter, an increase in the interfacial area for oxygen transfer, and enhanced oxygen transfer, especially at the larger scale.

The transfer efficiency dependence on depth has been assessed during clean water testing of the commercial size diffusers. Figure 3 presents standard oxygen transfer and standard aeration efficiencies for the EIMCO diffuser with respect to depth. The data indicates an increase in efficiency with increase in diffuser depth. Laboratory and pilot testing indicate that Bioleach systems perform better than clean water systems in terms of oxygen transfer efficiency and slightly worse in terms of standard aerator efficiency (due to mixing energy requirements of a slurry).

The effect of bioreactor design modifications on oxygen transfer is a complex problem and further complicated by the growth cycle of the bacteria. The scale-up approach being used by EIMCO involves holding the overall mass transfer coefficient (K_La) for oxygen constant and assuming that the reactor is well mixed.

MIXING REQUIREMENTS

Mixing Reynolds number has a significant impact on the oxygen mass transfer coefficient (K_La). Mixing in the EIMCO bioslurry reactor occurs primarily because of gas dispersion. Original pilot plant work and data obtained from the 6.5m (20 ft) deep reactors in Zimbabwe indicated that relatively stagnant regions existed within the reactor, particularly at the lower depths. To remedy this situation a high pumping, low shear axial flow hydrofoil has been mounted concentrically with the airlift shaft and is driven by an independent variable speed drive. The mechanical aspects of this type of design are well proven and have been adopted from the EIMCO Reactor-Clarifier™.

The dual drive concept has been tested side-by-side with a turbine mixed reactor in the EIMCO laboratory. Three impeller types have been tested in the turbine reactor, Rushton radial flow impeller' a pitched blade axial flow impeller' and a gas dispersing axial flow impeller. Impeller details are presented in Table 3.

The Rushton turbine worked effectively for gas dispersion, but had the disadvantage of high power consumption, high shear, and poor pumping characteristics. Bioleach kinetics were severely reduced at higher rotational speeds (330-350 rpm) and stopped altogether when the tip speed exceeded 180 m/min. Sludge buildup also prevented long-term continuous operations of the overflow reactor and the reactor had to be shut down after two months of operation for clean out.

The pitched blade impeller yielded poor kinetic rates and frequent inoculations of fresh biomass were required to keep the biological population active. The best impeller tested in the turbine reactor was the axial flow gas dispersing type. The kinetics possible with this reactor were equal to the kinetics obtained by the EIMCO design - however the power consumption was over 2 kw/m^3 as compared to the EIMCO power consumption of 0.3 kw/m^3. It must be pointed out that these tests were conducted at the 60 liter scale and power consumption cannot be directly compared to a full scale system. Estimated power consumption on a larger scale (100 -220 m^3) would range between 0.6 to 0.8 kw/m^3 for a turbine mixed system and less than 0.1 kw/m^3 for the EIMCO reactor.

SUMMARY

The EIMCO bioslurry reactor has been designed to meet the specific requirements of the bioleaching process in order to achieve production goals. Along with bioreactor scale-up issues, special attention has been paid to flowsheet development with respect to solid/liquids separation, waste liquor neutralization/stabilization, and biological cyanide destruction.

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TABLE 1

KINETIC DATA FOR BIOLEACH CIRCUIT OPERATING ON AN ARSENOPYRITE CONCENTRATE

Week	Stage	Average Oxidation Rate S gpl/day	Average Plant Oxidation Rate S g/l/day
1	1	4.2	
1	2	5.6	
1	3	1.3	3.7
2	1	3.4	
2	2	7.6	
2	3	2.9	4.6
3	1	3.4	
3	2	5.3	
3	3	2.4	3.7
4	1	4.9	
4	2	6.3	
4	3	4.4	5.2

TABLE 2

EXAMPLE OXYGEN DEMAND OVER ONE WEEK OF OPERATION

60-liter Continuous Train(a)

Bioreactor Stage #	Demand (Range) mg O_2/liter slurry/hour(b)
1	312-391
2	201-336
3	148-252

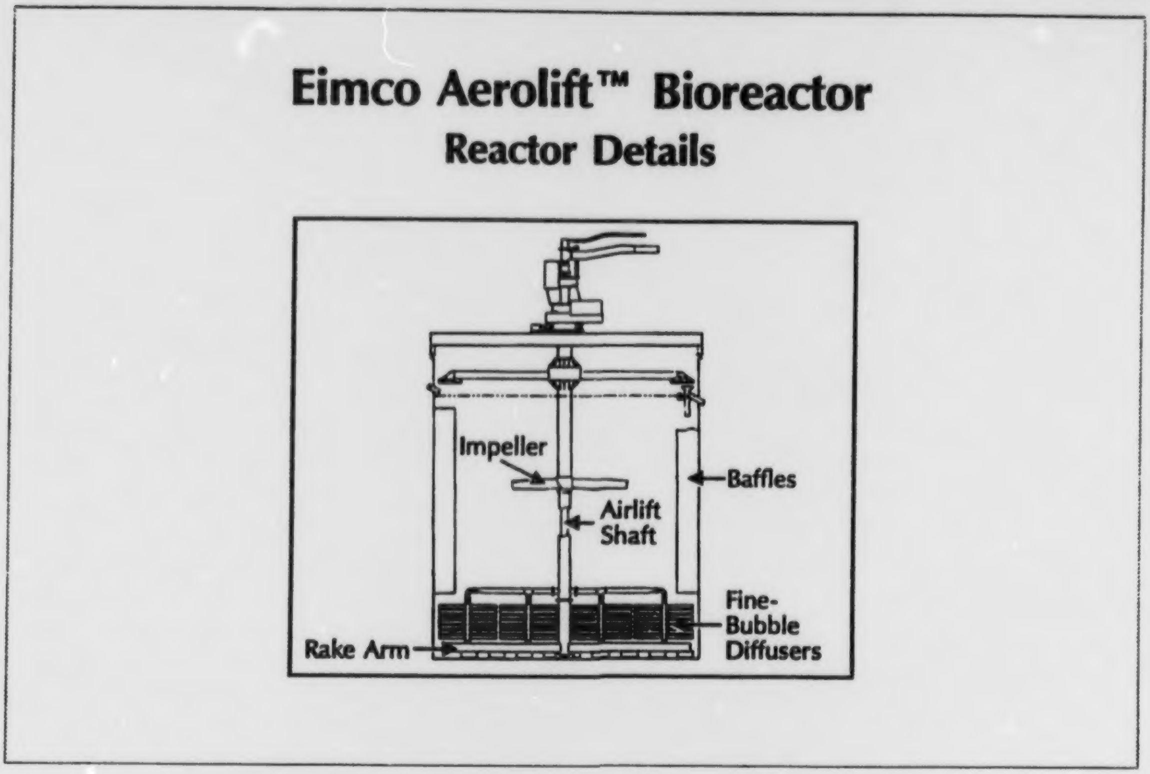
(a) Arsenopyrite concentrate

(b) Measured daily using an oxygen uptake method. YSI probe calibrated by air saturation method.

TABLE 3

BIOLEACH MIXING STUDY IMPELLER DETAILS

Bioreactor Design	Description	Diameter cm	Power #	Shear	RPM
EIMCO Bioslurry Axial flow	Hydrofoil;	25.4	0.3	Very low	10-80
Conventional	Flat blade: Radial flow	20.3	~5.6	High	160-400
	45° Pitched blade; Axial flow	15.2	~1.0	High	200-400
	Axial flow; Gas dispersing	25.4	1.0	Medium	350

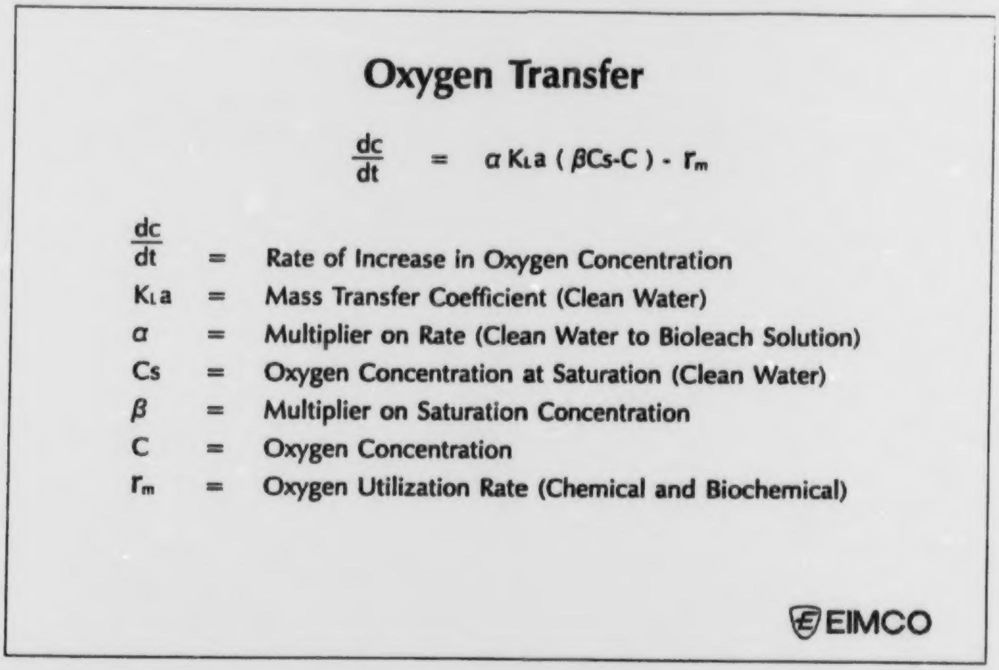


Eimco Aerolift™ Bioreactor

Reactor Details

Impeller
Baffles
Airlift Shaft
Fine-Bubble Diffusers
Rake Arm

Fig. 1 EIMCO Bioreactor Details



Oxygen Transfer

$$\frac{dc}{dt} = \alpha K_L a \left(\beta C_s - C \right) - r_m$$

$\frac{dc}{dt}$ = Rate of Increase in Oxygen Concentration

$K_L a$ = Mass Transfer Coefficient (Clean Water)

α = Multiplier on Rate (Clean Water to Bioleach Solution)

C_s = Oxygen Concentration at Saturation (Clean Water)

β = Multiplier on Saturation Concentration

C = Oxygen Concentration

r_m = Oxygen Utilization Rate (Chemical and Biochemical)

EIMCO

Fig. 2 Oxygen Transfer Equation

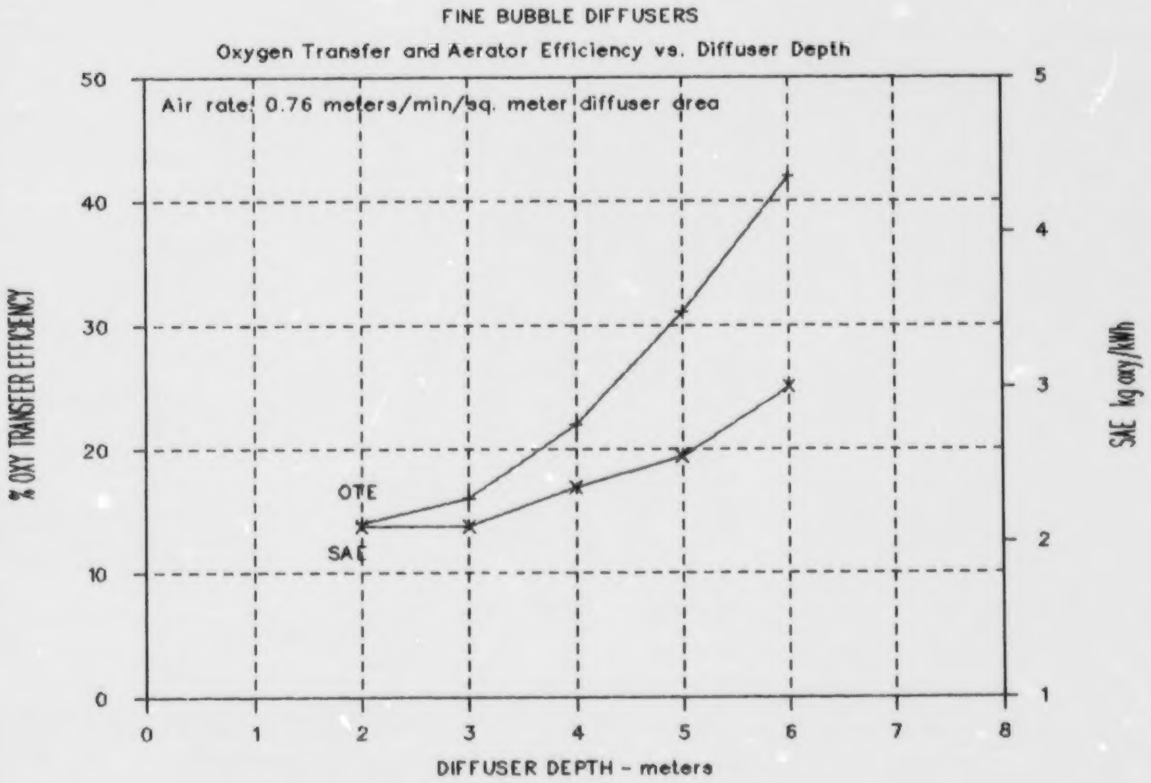


Fig. 3 Oxygen Transfer and Aerator Efficiency as a Function of Depth

USE OF A MODERATELY THERMOPHILIC BACTERIAL CULTURE FOR THE TREATMENT OF REFRACTORY ARSENOPYRITE CONCENTRATE.

*P.A.Spencer *, J.R. Budden * and R. Sneyd⁺.*

** Transworld Mining and Minerals (Australia) Pty. Ltd.*
+ Minproc Engineers Pty. Ltd., Brisbane.

A moderately thermophilic bacterial culture has been used in the treatment of a refractory arsenopyrite culture. The culture had a wider range of operating temperatures than *Thiobacillus* cultures and was more suited to the high summer temperatures experienced in Australia. Laboratory testing showed the thermophilic bacteria could operate under conditions similar to those used for *Thiobacillus* cultures and could treat a large range of sulphide samples. The optimum bio-oxidation conditions developed in the laboratory were tested on a commercial concentrate at pilot plant scale for 7 months without a loss of bacterial activity. The culture was able to maintain the optimum oxidation rate over the range 30 to 49°C and could tolerate very high arsenic concentrations (25 g/L) without affecting the oxidation rate. The data generated during the pilot plant operation was used to complete an engineering design study for a commercial scale plant. Capital and operating cost estimates specific to this concentrate and its remote location are given.

UTILISATION D'UNE CULTURE DE BACTÉRIES MODÉRÉMENT THERMOPHILES POUR LE TRAITEMENT DE CONCENTRÉS D'ARSÉNOPYRITE RÉFRACTAIRE

P.A. Spencer, J.R. Budden*, et R. Sneyd+.*

** Transworld Mining and Minerals (Australia) Pty. Ltd.*
+ Minproc Engineers Pty. Ltd., Brisbane, Australia.

RÉSUMÉ

Une culture de bactéries modérément thermophiles a été utilisée pour le traitement de l'arsénopyrite réfractaire. La gamme de températures à laquelle ces bactéries sont efficaces est beaucoup plus grande que celle de *Thiobacillus;* elles sont donc mieux adaptées aux températures élevées des étés australiens. Les expériences en laboratoire montrent que les bactéries thermophiles peuvent être utilisées dans les mêmes conditions que *Thiobacillus* et peuvent traiter un grand nombre d'échantillons sulfurés. Les conditions optimales de biooxydation déterminées en laboratoire ont été vérifiées sur un concentré commercial dans une usine pilote pendant sept mois, sans qu'il y ait diminution de l'activité bactérienne. Le taux d'oxydation optimal est maintenu à des températures variant de 30 à 49°C et les bactéries peuvent tolérer des concentrations élevées d'arsenic (25 g/L) sans que cela n'altère le taux d'oxydation. Les données recueillies pendant les tests à l'usine pilote ont été utilisées pour compléter une étude de conception d'une usine commerciale. Un aperçu des coûts d'opération et des coûts en capital relatifs au traitement de ce concentré et à son emplacement retiré est donné.

INTRODUCTION

Considerable investigation has been conducted into the use of *Thiobacillus ferrooxidans* and mixed cultures containing these bacteria in the treatment of metal sulphide minerals. Thermophilic cultures have recently received greater interest than previously with most emphasis on *Sulpholobus* cultures. Moderately thermophilic cultures have received only minor attention by comparison despite having some potential advantages.

Moderately thermophilic bacterial cultures have received far less attention than either *Thiobacillus* or *Sulpholobus* cultures but in some cases have been shown to be capable of treating a similar range of sulphide minerals with the advantage of higher metals resistance (Brierley, 1987). The moderately thermophilic culture used in this work has previously been shown to be able to treat a range of minerals and release the metals for recovery (J. Barrett *et al*, 1988). The conditions, with the exception of temperature, were very similar to those used for *Thiobacillus ferrooxidans*. The temperature range for maximum iron oxidation was found to be 40°C and 45°C with reasonable rates in the range 30°C to 49°C as shown in Figure 1 (P.A. Spencer, 1988). The culture was capable of sustaining the maximum bio-oxidation rate for an arsenopyrite concentrate in solutions with arsenic concentrations up to 25g/L arsenic. This moderately thermophilic bacteria has benefits in hotter regions because the need for expensive reactor cooling is reduced or removed entirely. Higher percentage solids and therefore smaller plant sizes are made possible by the high oxidation rates at high metals concentrations. Heating should be minimal, particularly in warmer climates.

There have been many reports on laboratory and even pilot plant tests using *Thiobacillus* cultures but there has been limited emphasis on how the results might be applied to the design of a commercial bio-oxidation plant. To date there have not been any reported applications of thermophilic cultures other than at a small laboratory bench scale. This paper describes the use of a moderately thermophilic culture for the bio-oxidation of a commercial arsenopyrite concentrate and indicates how the laboratory data can be applied to the design of a commercial bio-oxidation plant. Estimated capital and operating costs have been calculated.

PROCEDURES

The sample used in this case study was a commercial arsenopyrite concentrate from an operating mine in Western Australia. Ambient summer temperatures in the mine locality exceed 45°C in most years. The concentrate assayed 16.7% arsenic, 28% iron, 34% sulphur and 50g/t gold. Nickel assays were variable but could be as high as 1.2%. The arsenopyrite concentrate was 80% passing 45 microns and further grinding was not normally necessary. The gold was extremely fine and could not be liberated even by grinding to 100% passing 5 microns (R. Dunne *et al*, 1988).

The culture was maintained at 1 to 5% solids in a 9 liter air agitated pachuca reactor. Inoculum was removed as needed for the stirred reactor tests.

All testing was performed in the temperature range 40°C to 45°C when measured in the slurry.

Batch Testing

Batch bio-oxidation tests were given a 10% v/v inoculum with the balance of the volume being added as pH adjusted nutrient solution. The reactor was allowed to reach temperature before the sample was introduced. All solids densities have been expressed as % weight/volume.

The bio-oxidation progress was monitored by measuring the soluble iron and arsenic, by measuring Eh and by measuring oxygen uptake rates. The pH was reasonably static due to the ion exchange properties of some of the clay minerals in the concentrate.

At the completion of the oxidation, the solids were separated from the bio-oxidation solution and leached in cyanide solutions. CIL leaching was not considered as the mine had an existing CIP plant.

Continuous Testing

The continuous testing was performed over a twelve month period in a number of multistage reactor systems up to a 500 liter pilot plant scale. The dried concentrate was added using a spiral feeder, where possible, to simulate the anticipated plant practice. Nutrient addition, pH adjustment and feed addition were made directly into the first bio-oxidation stage.

The continuous system was monitored in the same manner as that used for the batch tests. The oxidized solids were collected and a composite prepared for later gold extraction testing.

A recycle option was tested to determine its effect on the bio-oxidation rate. Slurry from the last stage was recirculated to the first stage at an amount up to 50% of the total flow.

RESULTS

Batch Testing

A number of batch tests were completed in shake flasks to determine whether standard bio-oxidation conditions could be applied to the arsenopyrite concentrate. The results in Figure 2 show a significant lag period was observed for standard conditions but this was removed by slightly altering the nutrient blend. No rate data suitable for designing a commercial plant can be obtained from the shake flask type of testing. Rates in this type of test are low due to restricted aeration and solids density.

Batch testing in agitated reactors with positive aeration was used to identify the optimum solids density for arsenic bio-oxidation. Figure 3 shows the arsenic oxidation rate per unit of reactor volume as a function of the solids density. It was found that the bio-oxidation rate increased with solids density up to 15% w/v but was no improvement was obtained at 20% w/v solids. This oxidation rate per unit of reactor volume is important in determining the volume of the reactors needed to treat a given tonnage of concentrate per unit time. The total volume for the bio-oxidation plant can be estimated using the tonnage, the arsenic content and the bio-oxidation rate instead of using empirical testing in a continuous test system where the solids density was gradually increased and the residence time reduced until the gold extraction criteria was no longer satisfied. Continuous testing conditions were thus more closely identified before the testing commenced.

Other batch tests were used to produce oxidized residues with different levels of arsenic oxidation. These oxidized residues were leached with cyanide solutions to extract the gold and obtain a correlation between arsenic oxidation and gold extraction. The correlation obtained for this arsenopyrite sample is shown in Figure 4. Almost complete arsenic oxidation was required for gold extractions greater than 90%.

Continuous Testing

The continuous testing was planned using the oxidation rate obtained for the batch testing at 15% w/v solids and the arsenic content of 16.7 percent. These parameters plus the reactor volume for the continuous system and a residence time of 7 days should have achieved between 70 and 80% arsenic oxidation. The gold extraction expected for these levels of bio-oxidation was between 75% and 80%. These levels were chosen so that the continuous data would fall within the ranges established by the batch testing.

Figure 5 shows the arsenic oxidation rate per unit of reactor volume as a function of elapsed time for 15% w/v solids density. The arsenic oxidation rate for the continuous system was within the range expected from the batch testing after 3 residence times. The arsenic oxidation level achieved for the same time period is shown in Figure 6. The arsenic oxidation also reached the expected level within 3 residence times of commencing continuous operation. An average oxidation level of 78.9% was achieved over the period of this continuous test. These results demonstrated that the bio-oxidation response for a given range of conditions can be predicted from systematic batch data and confirmed in a minimum of time using an appropriate continuous system.

The arsenic oxidation rate was not affected by the arsenic concentration over the range of concentrations which existed in the continuous system. Figure 7 shows the arsenic oxidation rate as a function of the discharge arsenic concentration from the system over more than 1000 hours of testing. Similar oxidation rates were obtained at the higher arsenic concentrations to those obtained at 15000ppm arsenic.Oxygen uptake rates were measured during the continuous operation. The uptake rates for each of the bio- oxidation stages have been presented in Figure 8. The oxygen uptake rate can be used in conjunction with the efficiency to calculate the aeration rates required for each stage of the commercial operation.

The slurry recycle was found to be detrimental to the arsenic oxidation rate and the percentage arsenic oxidation as shown in Figure 9. The rate and therefore the level of oxidation achieved in the continuous system was significantly lower when the recycle was operated. Reasons for the lower oxidation rate were not identified at the time of the testing.

Solution separated from the oxidized solids was neutralized with lime or limestone to precipitate basic ferric arsenate. The reagent requirements were used to calculate the costs for neutralization.

The neutralized solids from bio-oxidation were leached with cyanide to obtain gold extraction data for the continuous bio-oxidation residues. The data has been presented in Figure 10 and compared with the correlation for the batch testing. The results for the residues from continuous bio-oxidation agreed closely with the batch test data.

Estimation of Capital and Operating Costs

The data generated from the batch and continuous testing has been used to estimate the capital and operating costs for a bio-oxidation treatment stage for the arsenopyrite concentrate examined. The concentrate was to be processed at approximately 13,000 tonnes per annum with an average arsenic content of 14% and 70 g/t gold for the life of mine. The contained gold value per annum was calculated at A$ 14.28 million. The capital costs were estimated to be A$ 2.5 million. The operating costs were estimated to be A$ 36 per ounce of gold recovered (selling price $488 per ounce June 1989) or A$ 80 per tonne of concentrate. The contribution of the various plant parameters to the estimated operating costs for bio-oxidation have been shown in Figure 11. A large proportion of the costs was related to the neutralization of the arsenic laden bio-oxidation solution. The high arsenic content of the concentrate also had a large impact on roaster costs (not given).

CONCLUSIONS

The moderately thermophilic bacterial culture developed by Transworld Mining and Minerals (Australia) Pty Ltd was capable of treating an arsenopyrite concentrate at high solution arsenic concentrations without resorting to insitu precipitation or interstage solution removal. The culture has been maintained and used for bio-oxidation testing at temperatures in excess of 40°C for more than twelve months showing that the culture could withstand the high ambient summer temperatures found in some gold mining regions.

The laboratory batch data was used to establish the optimum operating conditions for the continuous test system. The continuous testing could thus produce data on the residence time, oxygen uptake rates and gold extraction from the bio-oxidized residues without having to empirically arrive at the optimum continuous bio-oxidation conditions by trial and error. The data on residence time, oxygen uptake rate, solids density and neutralization was used to estimate the capital and operating costs to treat a given tonnage of the arsenopyrite concentrate.

The estimated capital cost for the bio-oxidation plant was approximately 17.5% of the value of the annual contained gold. The estimated operating costs per ounce were 7.4% of the gold price. Other costs for the more conventional unit operations would have to be very high to prevent the bio-oxidation treatment and CIP gold extraction from being viable.

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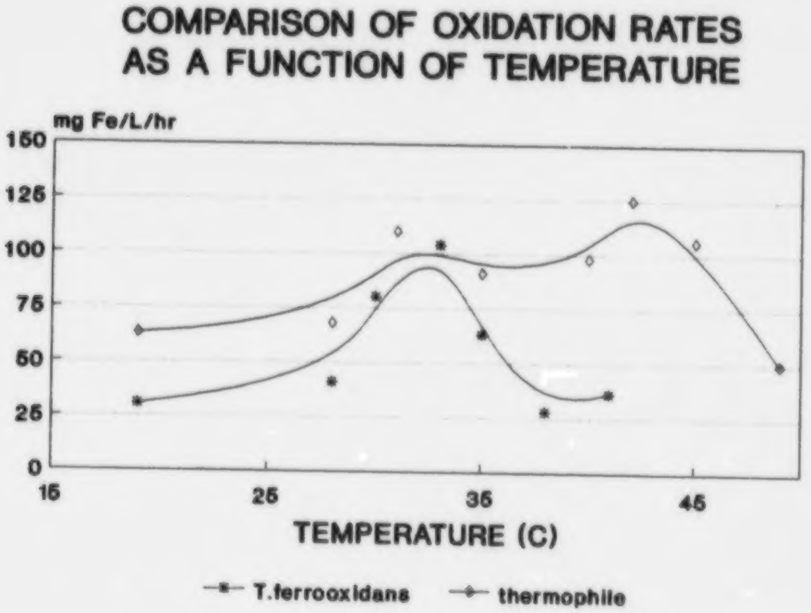

Fig. 1 Comparison of Oxidation Rate for *T. ferrooxidans* and Thermophilic Culture for a Range of Temperatures.

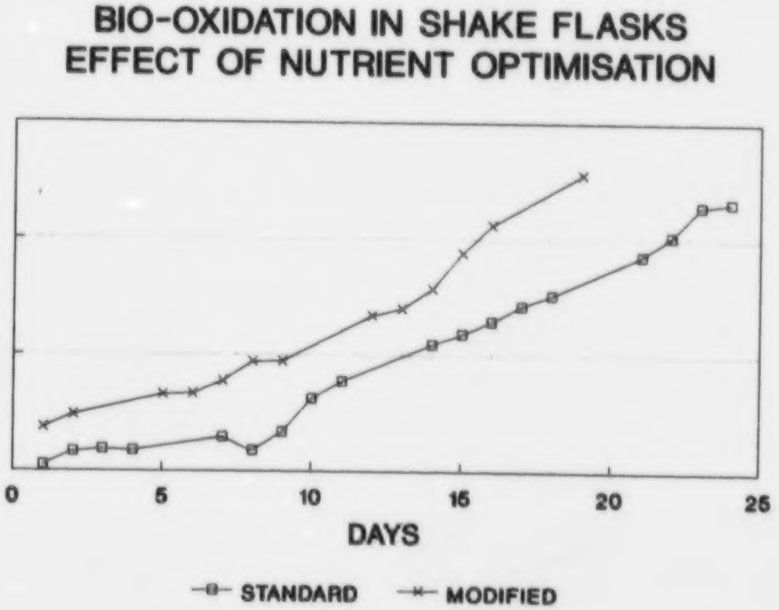

Fig. 2 Shake Flask Test Results Showing Effect of Nutrient Optimisation on Oxidation Performance.

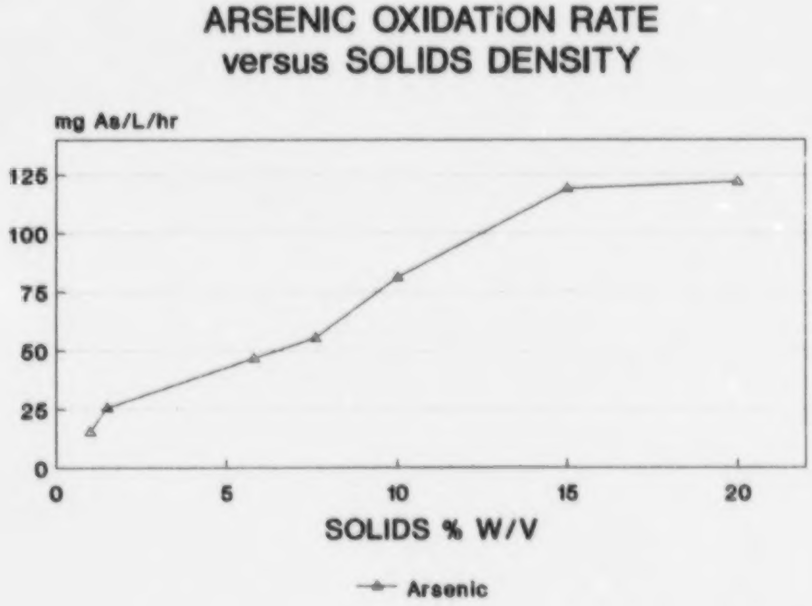

Fig. 3 Arsenic Bio-oxidation Rate as a Function of Solids Density.

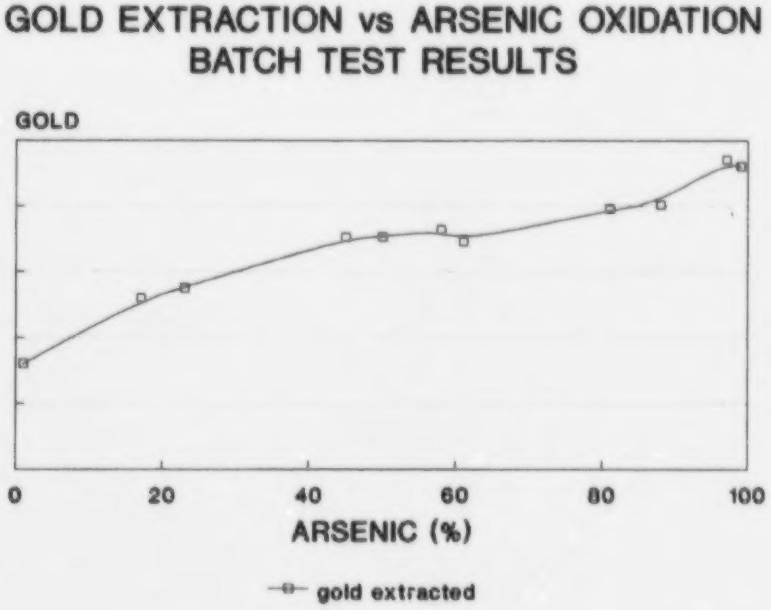

Fig. 4 Gold Extraction as a Function of Arsenic Oxidation in Batch Testing.

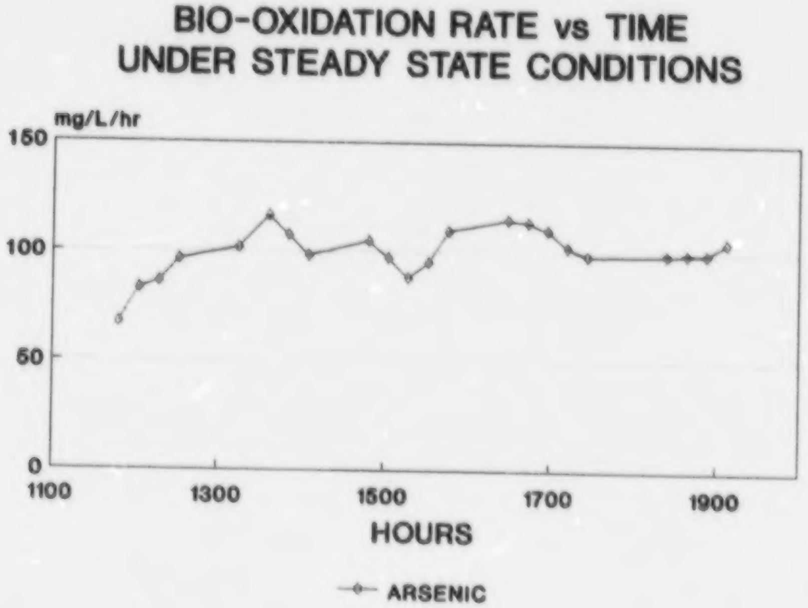

Fig. 5 Percentage Arsenic Oxidation in Continuous System under Steady State Conditions at 15% Solids.

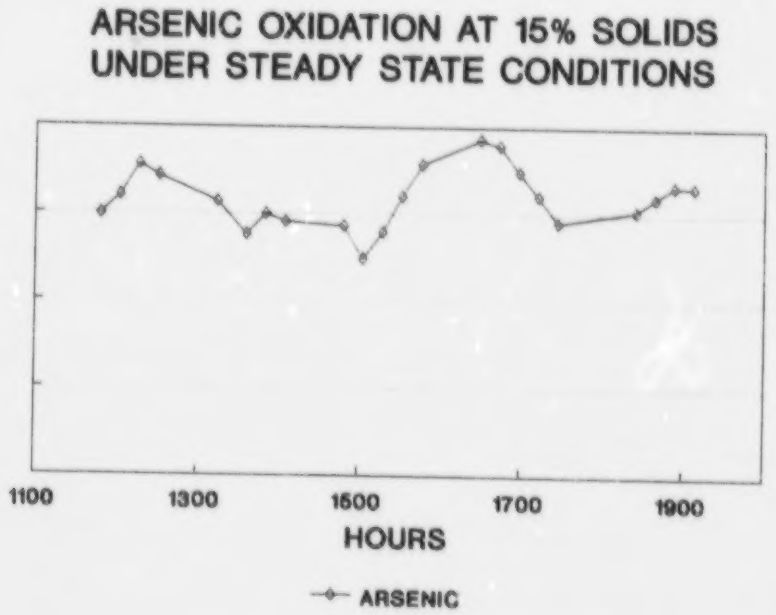

Fig. 6 Bio-oxidation Rate for Arsenic in Continuous System under Steady State Conditions at 15% Solids.

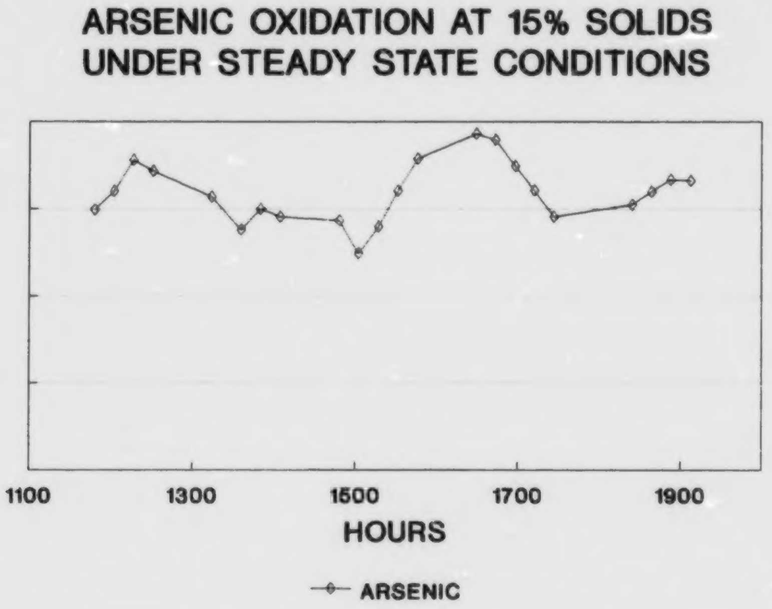

Fig. 7 Bio-oxidation Rate of Arsenic as a Function of Arsenic Concentration in Continuous System under Steady State.

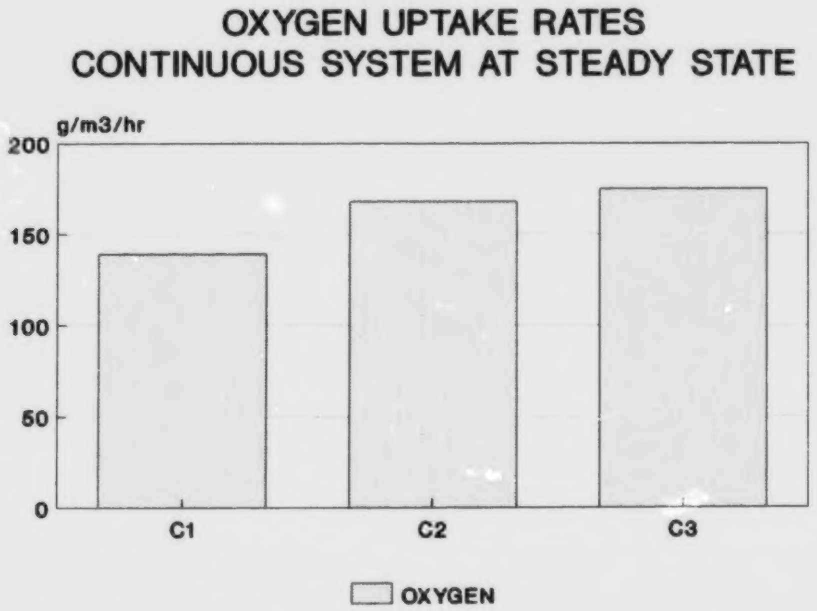

Fig. 8 Oxygen Uptake Rates in the Three Stages of Continuous System at 15% Solids.

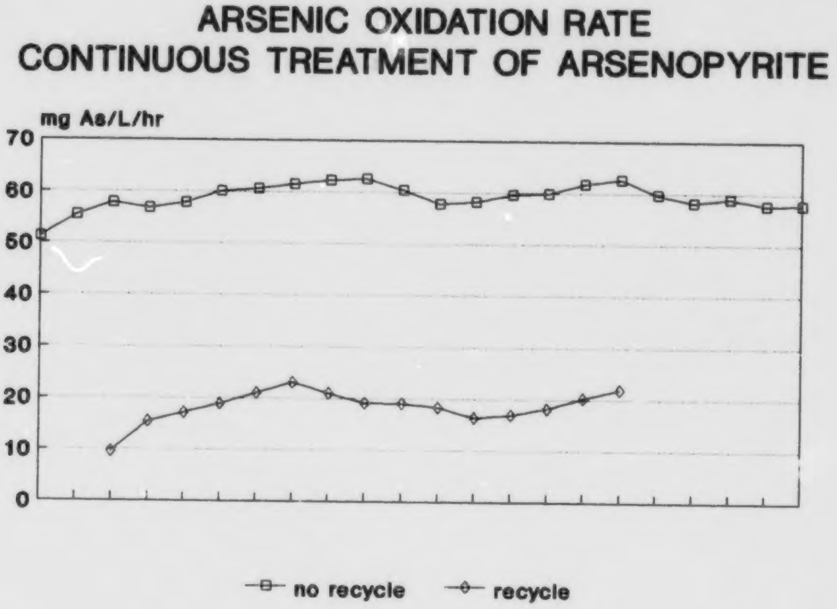

Fig. 9 Arsenic Oxidation Rate with Slurry Recycle Compared with Standard Continuous Operation.

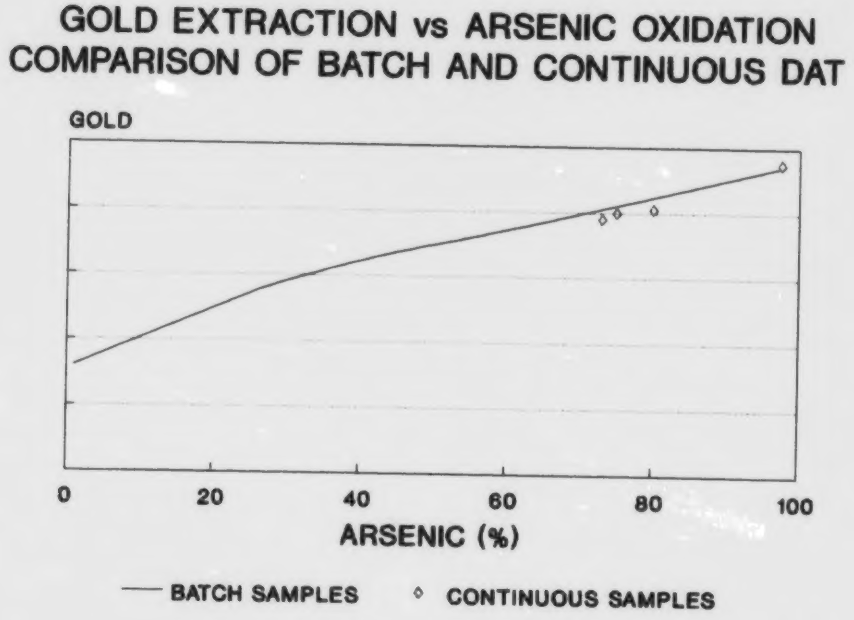

Fig. 10 Gold Extraction Data for the Continuous Bio-oxidation Residues Compared with Batch Data.

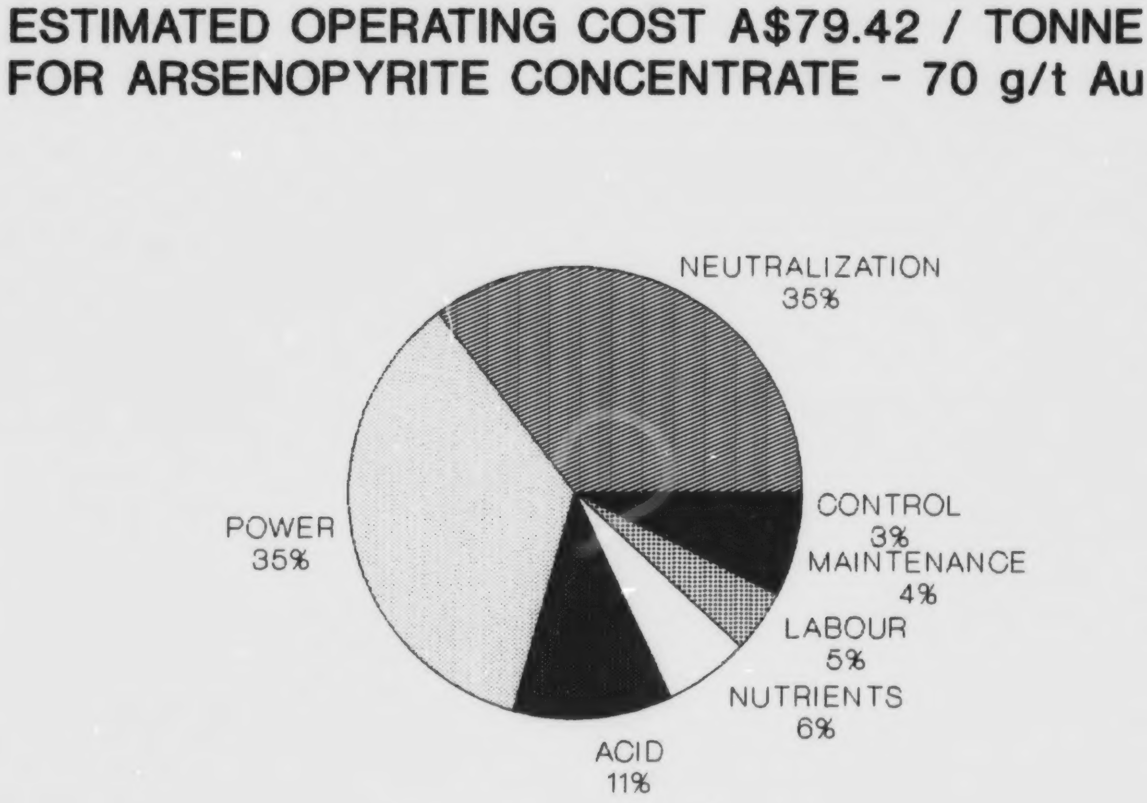

Fig. 11 Distribution of Estimated Operating Costs for Bio-oxidation Plant Parameters.

DESTRUCTION OF HUMIC ACIDS AND ORGANICS IN MINERAL DEPOSITS BY MICROBIAL ACTION — A CRITICAL REVIEW

V.I. Lakshmanan, J. Christison and P.D. Tackaberry
ORTECH International, Mississauga, Ontario Canada
and
R.G. McCready
CANMET, Ottawa, Ontario Canada

ABSTRACT

Humic acid and organics cause various problems in mining industry unit processes and are most significant in the aluminum processing industry. The degradation of organics in the Bayer liquor produces high concentrations of oxalic acid converted to sodium oxalate causing liquid/solid separation problems and increased NaOH consumption. Due to the strong alkalinity of the Bayer liquor, microbial pretreatment of bauxite ore may be more feasible, and a conceptual flowsheet has been developed.

DESTRUCTION DES ACIDES HUMIQUES ET D'AUTRES MATIÈRES ORGANIQUES DANS LES DÉPÛTS DE MINERAI PAR ACTION MICROBIENNE — ÉTUDE CRITIQUE

V.I. Lakshmanan, J. Christison et P.D. Tackaberry

ORTECH International, Mississauga, (Ontario), Canada

et

R.G. McCready

CANMET, Ottawa, Ontario Canada

RÉSUMÉ

Les acides humiques et les matières organiques peuvent causer divers problèmes dans l'industrie minière, surtout dans le traitement de l'aluminium. La dégradation des matières organiques dans le liquide de Bayer peuvent produire des concentrations élevées d'acide oxalique, acide qui est ensuite transformé en oxalate de sodium, ce qui peut causer un problème de séparation des phases liquides et solides et augmenter la consommation de NaOH. En raison de l'alcalinité élevée du liquide de Bayer, il peut être possible de faire un prétraitement microbien de la bauxite; un schéma fonctionnel a donc été établi.

The Nature of Humic Substances

Humic substances are natural organic compounds formed from the partial decomposition of plant and animal matter and as such are the most extensively distributed natural organic matter on earth. In considering the definition and nature of humus, it is not only the amorphous brown- black material in the soil, but also the colourless to light coloured waxes, resins, hydrocarbons, nitrogenous compounds, etc. that are typically associated with the humus.

Humification has been postulated to occur through a number of different methods although generally is a result of microbial activity more so than chemical transformation.

A lignin derived process whereby redox modified lignin condenses with available proteins (Fig. 1, Pathway 4) represents the classical theory as developed by Waksman (1932). A more accepted modern theory as proposed by Stevenson (1982) is the polyphenol modification (Figs. 2 and 3). Here, lignin still plays the primary role, but phenolic decomposition products released during microbiological attack undergo enzymatic conversion to quinones which further polymerize in the presence or absence of amino compounds to form humic like macromolecules (Fig. 1, Pathway 3). A somewhat similar origin (Fig. 1, Pathway 2) has polyphenols synthesized by microorganisms from nonlignin carbon sources and then enzymatically oxidized to quinones with further conversion to humic substances as described above. Nonenzymatic browning reactions between sugars and amino acids may also be responsible for humic substance production. It is most likely that all four mechanisms of humus production occur at any one time, the determining factor being the environment the formation is taking place in, aquatic or terrestrial.

The fundamental structure of humic substances is often variable but certain facts have been established. Four fractions have been isolated based on acid/alkali solubility: Fulvic(FA), Humic(HA), Hymatomelanic Acid and Humin with increasing refractories as listed (Schnitzer and Khan, 1974). Fulvic acid has been characterized as having acid and alkali solubility, molecular weight by gel filtration of between 2000 and 9000, and considerable heterogeneity, and a partial molecular structure has been proposed by Schnitzer and Khan (1978) (Fig. 5).

Humic Acid (Fig. 4) is thought to attain more complexity through chemical or physical condensation reactions involving peptides, carbohydrates, metals and phenolic acids as in Figure 6 (Schnitzer and Khan, 1972). It is alkali soluble with molecular weight ranging from 5000 to 100,000 (Hurst Burgess, 1976). Hymatomelanic Acid is soluble in alkali and ethanol and Humin is soluble in alkali and acid, both consisting of relatively unchanged organic material and truly humified material, and humic acids closely complexed with clay minerals. Elemental composition (C,H,O,N,S) has considerable variability in fraction percentages and further variability depending on the aquatic or terrestrial origin of the humus.

Industrial Problems

Humic substances are ubiquitous in the environment and as such cause problems in the aluminum (Al) and uranium (U) processing industries. Adverse effects are seen in various unit operations including flotation, solid/liquid separation, ion exchange (IX) and solvent extraction (SX). The humic substances causing these effects are present in the ore itself or the process water used in the plant operation.

The most serious effect humic substances have is during Al processing. Bauxites contain from 0.1-0.6% organic C, most of it occurring as humic substances (Lever, 1978). Upon Bayer process digestion over 50% of the organic C is extracted into the liquor, the concentration building to an equilibrium saturation point with recycling to 7.25 g carbon/L. Characterization of these organics has identified about 25% high molecular weight (>500) freshly extracted material, 25% intermediate degradation products containing the building blocks of large humic molecules (benzene carboxylic acid, phenolic acids) and about 50% low molecular weight degradation products (formate, acetate, oxalate, succinate). It is the latter group creating the most problems, causing poor classification — settling of the fine Bayer seeds, and excessive scaling in process lines and tanks. Problems caused by the high molecular weight humics are liquor foaming, deactivation of Bayer hydrate and oxalate seed, and increased alumina and oxalate solubilities in the pregnant liquor. Other significant process problems (Guthrie, et al, 1979) include lower alumina yield, generation of excessively fine aluminum trihydroxide particles, lower red mud settling rates and loss of caustic due to the formation of sodium organic compounds.

In the uranium industry, problems are confined to those ores of a sedimentary nature where humic substances are present in sand, siltstone and carbonaceous deposits (McDonald *et al*, 1980). Humic contaminants extracted into the SX organic diluent are responsible for emulsion and crud problems from humic degradation products forming insoluble salts (Ritcey, 1980). IX is adversely affected as well by organic contamination of the pregnant leach solution where IX resins can experience decreased reaction sites for U-adsorption.

Other mineral processing operations affected by humic substances are those which use humic contaminated process water. There has been seasonal problems reported by Kidd Creek in the pyrrhotite flotation circuit, while Kennecott has shown that humus organics adversely effect the flotation recovery of molybdenum. Pregnant liquor robbing of gold by contaminant humics has also been reported by Mintek.

Biodegradation

The biological role in humus formation may be particularly significant in why humics are so resistant to breakdown. By their nature, humics help the organisms which produce them to resist biological decay, i.e. they are designed to be resistant to biodecay. Phenols, quinones, phenol carboxylic acids and phenol glucosides are among those compounds which act in a preservative capacity in humus environments (Swain, 1963). The dynamic equilibrium factor is also significant in that degradation products after a short period of time appear to reform into humic-like substances.

Estimates for the time required for humic components to degrade in the natural environment have been calculated by radiodating (Fukushima, 1982). They are:

Freshly formed humus	2-6 years
Fulvic Acid	40-120 years
Humic Acids	80-2000 years

In the case of ore deposits the predominant humic substances will be of the more mature age group and therefore exhibit the recalcitrance commonly observed.

In seeking biological procedures to degrade humic substances, it is important to define accurately the nature of the problem. Through its controlled degradation, organic nitrogen bound-up in the molecular structure is gradually made available to vegetation. The resistance to degradation of humic substances is obviously a factor in the control of the natural process. That they degrade is well recognized but as Flaig *et al.* (1975) commented about humic substances "they serve as carbon source only for special species of organisms." A goal of this study is to identify this special group of organisms and examine what is known about how they degrade humic substances. One perceived strategy will be to find a method to overcome the natural barrier against microbial degradation which is inherent in the molecular structure of humic substances.

Fungi

The isolation of fungi capable of actively decomposing humic acid (Table 1) was reported by Burgess and Latter (1960) and was significant from the fact that there was evidence of co-metabolism. They found that 33% and 42% of a 0.12(w/v) humic acid preparation was degraded by *Spicaria* sp. and *Polysticus* sp. respectively, but only in the presence of 2% sucrose. This energy-dependant degradation is closely related to kraft mill effluent decolorization by lignin utilizing fungi in that the mechanisms postulated for the biologically catalyzed degradation of lignin may be of interest when the mechanisms of degradation of humic substances are considered.

Biodegradation of HA and FA, based on the mycelium yields (g/unit volume) of ten different fungal species, increased in the presence of 1.5% glucose and 0.15% nitrate. Fulvic acid was being used as a N- source and generally better utilized than humic acid although HA was used more uniformly (Dubrovska, Macor, 1978). Macor (1979) using the same group of micromycete fungi plus four other species (Table 1) reported that the intensity of utilization was dependant on the fraction of FA or HA used as well as the fungal species. A higher dry weight of mycelium was contained with FA as the sole C & N sources possibly because the fulvic acids are smaller molecules and less condensed than the humic acids. Both FA and HA stimulated dry weight mycelium production when added to the nutrient complete Czapek-dox medium.

Supplemental C and N sources were required to support the growth of 3 fungal isolates (Khandelwal and Gaur, 1980) and resulted in between 24% and 40% degradation of 0.1% HA in 40 days (18% on soil humates) (Table 1). These results were obtained on a fairly fresh manure humus and perhaps indicate the higher degree of aromaticity in soil environment.

Replacement culture technique using *Poria subacida* helped to demonstrate that the loss of colour in liquid FA (0.05%) culture was due to degradation and not due to adsorption onto the mycelium (Mathur, 1969). The enzyme phenol oxidase was shown here to be important in later stage fulvic acid breakdown as heating caused the loss of this property. Mathur and Paul (1967) has earlier shown that a replacement-shake culture of *Penicillium frequentans* utilized 35% of the humic acid and that the fungus preferred the aromatic moiety of the humic complex. Shaking inhibition of decolorization was noted in the above work (Mathur, 1969) as was the degrading activity of *Marasmius oreades* on HA and FA (Mathur, 1970). After one day in replacement culture 44% of the 0.05% FA and 15% of the 0.05% HA was degraded by the basidiomycete fairy ring mushroom (All Table 1).

A mechanism of degradation of humic acid was postulated through the observed fungal ability of 29 strains to decolorize HA. Hurst (1962) found a positive correlation between the ability to decolorize HA and the presence of reducing ability on the aromatic carboxyl group. They further went on to postulate that humic acid adsorption on to the fungal hyphae was necessary where the presence of NAD(P)H and enzymes would make the reaction possible. Based on these results they suggested that the initial reaction with HA involved the reduction of the carboxylic group(s) between aromatic monomers. However, in view of the aerobic growth of the organisms, the reductive capacity was considered to be a secondary reaction of the aerobic metabolism of the other carbon source in the medium (i.e. 0.5% glucose).

Humic acid used as a sole source of carbon was most efficiently bio-degraded. This was followed by humic acid used a C and N source. (Mishra, Srivistava, 1986). The fungi *Aspergillis awamori, Humicola insolense* and a *Penicillium* sp. were most effective. The unavailability of the humic acid N is thought to be responsible for the inefficient use of HA as a sole N source to these microorganisms.

There appears to be strong evidence that the degradation of humic substances by fungi is dependant for optimum effect on the presence of alternative energy sources (i.e. carbon nutrients) in the culture medium. In some cases, the presence of a nitrogen compound is also beneficial. Thus the degradation of humic substances is a secondary reaction of the cells metabolic pathways. The significant variations between the abilities of different species and different strains of the same species to degrade humic substances may reflect the abilities of the respective organisms to utilize C and N compounds, i.e. biopolymers entrapped within the humic substances.

Actinomycetes and Bacteria

Information on the degradation of the humic substances by actinomycetes is summarized in Tables 2 and 3. Actinomycetes are classified as autochthanous soil microorganisms and as such, selected species have been implicated in the decomposition of lignin and the reader is directed to separate reviews. (Crawford and Crawford, 1984; Amer and Drew, 1980)

In view of numerous reports implicating actinomycetes in the degradation of soil humus (Federov and Il'na, 1963; Volkova, 1961) Mathur and Paul (1967) found that of 30 soil actinomycetes tested none could effectively utilize HA, although one, an *Arthrobacter* sp., utilized 11.5% (of a 0.2% w/v HA preparation) based on colour loss in Czapek-Dox medium without C or N supplementation.

Debosz and Golebiowska (1975) found that in aerated pure cultures of actinomycetes, HA synthesis was favoured over decomposition. In mixed soil cultures the addition of glucose as sole supplement resulted in degradation while glucose and N-supplement resulted in HA synthesis. Ivanova (1979) showed that a N-supplement accelerated straw decomposition and the rate of humus formation, and identified the actinomycetes no further than members of the Proactinomyces and Micromonosporaceae.

Arthrobacter globiformis degraded fulvic acid (Rifai and Bertru, 1980). The presence of benzoate and lactate appeared to stimulate the degradation process, possibly due to their enhancement of the production of extracellular enzymes able to hydrolyze aromatic groups.

Khandelwal and Gaur (1980) observed a 2-3 times increase in humate utilization with C and N supplementation, although they note the possible conversion of low molecular weight compounds to humic acid.

Monib et al (1981) compared the HA degrading abilities of 28 *Streptomyces* species by monitoring CO_2 release over 46 days. All the *Streptomyces* sp. used 0.2% (v/v) HA as a C-source ranging from 11-21% utilization, however, 25-66% (of the 11-21%) occurred in the first 3 days

A closely related group to the actinomycete are the other procaroytes, the bacteria. The Pseudomonads and *Bacillus* sp. have been noted as most predominant in humus degradation but recently a number of other species have been implicated (Table 4).

Mishustin and Nikitin (1961) observed the ability of a *Pseudomonas* sp. to decolorize HA and further was thought to involve oxidation by peroxidase.

Mathur and Paul (1967) found that an unidentified *Pseudomonas* sp. and a *Bacillus* sp. utilized about 15% and 11%, respectively, of a 0.2% HA preparation while an *Agrobacterium* sp. utilized the HA as both C and N source.

Pseudomonas sp. were the most active humus decomposers, but *Bacillus* sp., *Nocardia* sp. and *Actinomyces* sp. all had redox enzymes dehydrogenase and peroxidase present (Teslinova, 1979).

Microbial attack against the aromatic structure in fulvic acid (Kunc et al, 1976) was concluded after bacterial numbers increased as did the ability to oxidize vanillic and p-hydroxybenzoic acids.

A mixed culture of *Pseudomonas maltophila* from a forest soil was capable of using HA as a carbon and energy source (Swift et al, 1987). Degradation was evident in 2-6 weeks of culture with the molecular weight distribution shifting to the lower molecular weight compounds.

Intensive anaerobic humic substance mineralization activity was associated with purinolytic *Clostridium* sp. (Emstev, 1985), *C. sartagoformum* and *C. paraputrificum* were both very active in the decomposition of the aliphatic and core parts of the molecule utilizing the humus as N-source. Proteolytic and saccharolytic species decompose only the peripheral parts of the molecule without breaking the aromatic nucleus as the purinolytic species would.

Resistance to biodegradation was evident from an absence of changes in molecular weight distribution (Blondeau, 1988). A heterotrophic soil bacterial population initially increased in numbers when grown on HA containing media but after 60 days decreased to the inoculum size. The presence of carboxylic acids increased especially during anaerobiosis.

Although numerous examples exist on the degradation of humus, a number of researchers note the reformation of humic substances within the same work, especially with Actinomycetes. Bacteria tend to be associated with the degradative aspect of humus but incubation times are considerably lengthy. It is obviously a complex situation when both C and N supplementation stimulates both synthesis and degradation.

Conceptual Flowsheet

Because of the extreme chemical environment of the Bayer liquor and the U-rich leach solutions, the removal of humic substances is thought to be best approached before the raw material enters the process stream. As most biological processes occur near pH 7, treatment of ores is most amenable at a stage where selected organisms will thrive on the humic substrates. A hypothetical process flowsheet is shown in Figure 7. The process can be performed either at the mine site or at the refinery. Five unit processes or process streams are identified and discussed below:

1) Run of the Mine Ore

Since the goal is to remove those substances responsible for giving rise to oxalate, other aliphatic acids and humic substances, the important question is which compounds must be removed. The desirable information could include the precise characterization of the humic and other organic substances as they occur in an ore at any one site being investigated. Since biological processes are noted for their specificity, the above information could be helpful in identifying the target compounds in the crude ore.

2) Crushing and Sizing

Assuming that a microorganism is being sought to actively degrade the humic/organic components, studies can be undertaken with the ore to ascertain the degree of particle size needed to expose the humics and organics versus additional cost of size reduction and materials handling. The permeability of the ore will also need investigation.

3) Selection of Microorganism

The ability of selected fungi, actinomycetes and bacteria to degrade the lower molecular weight fractions of humus (HA or FA) has been noted. Conversion factors as high as 80% (rare) were reported. A substantial screening program to enrichment culture approach will be required to identify these organisms with the greatest ability to degrade the organic materials of interest. This search may be facilitated if the organics can be isolated in sufficient quantity from the ore to allow use as substrate in the microbial selection process. The ability of an individual organism to degrade humic fractions is both species and strain dependant so to achieve adequate rates and degree of degradation may require a mixed culture technique. Care will be needed here to ensure that organisms chosen do not cause recombination of humus-like pigments. Suitable inoculum development methods to introduce the organism(s) into the ore stockpile must be worked into the process design. A possible solution to maintain an adequate microbial population will be to recycle organisms in the leachate, or the use of alternate nutrient solutions as long as the ability of the organisms to attack the organics is retained.

4) Culture Conditions

Factors to be considered will include control of pH, temperature, moisture and level of aeration. Although most organisms noted in the literature are aerobic, excessive aeration can lead to the recondensation of phenolic type degradation products. Submerged or partially submerged culture technique may be required to control oxygen levels and keep the organisms in contact with the organics for an adequate length of time, or recycling and reaeration of the leach liquor in a heap leach may be satisfactory.

Temperature will be influenced by local environmental conditions and the optimum pH will reflect the requirements of the microorganisms used.

Organisms able to utilize the humic materials as their sole source of carbon and N may only require phosphorus or nitrogen supplements. Partial degradation of the humics to soluble fractions would enable removal in the effluent, therefore, total degradation may not be necessary. The use of a minimal level of added nutrients and the limitation that organisms in the culture must be able to utilize the humics as a C-source will also inhibit growth of contaminant organisms.

5) Culture Design

Any system designed must take into account relatively long incubation periods. The isolation of organisms able to more effectively degrade the organics may allow these periods to be reduced.

The particulates of the ore will influence the rate of the process. The presence of clay particles has been observed to decrease the rate of decomposition of FA and HA in liquid culture and a similar effect might be expected in the process. At the end of the treatment process, a chemical leaching with dilute alkali may be helpful in removing the adsorbed organics.

Water requirements are expected to be high, while at the same time continuous removal of the degraded humic materials is desirable to prevent reactions such as recondensation of the lower molecular weight phenolics. Removing the organics before the organisms metabolize them will also encourage more hydrolytic activity on the part of the organisms as they seek an energy source.

Conclusions

1. Humic substances are ubiquitous, organic compounds of varying molecular complexity occurring as a result of microbial more than chemical activity.
2. Humic contamination problems are encountered in mineral processing on various fronts. The aluminum industry is most seriously affected, where degradation products in the Bayer process liquor cause many problems. Pyrrhotite flotation, gold robbing, SX and IX contamination are also adversely affected.
3. The biodegradation of humics is seen as a slow, complex breakdown — synthesis cycle. Fungi present the greatest biodegradation potential, although the stimulatory effect of secondary C and N sources indicates co-metabolism. Actinomycetes and bacteria provide more complexity in this process with the former showing synthesis tendencies while in the latter anaerobic bacteria displayed higher than normal levels of degradation. In all three groups, species and strains showed a high degree of specificity for the different fractions of humus.
4. To successfully remove humic contaminants from mineral processes will require a separate unit operations, operating independently of the harsh chemical environment present in most affected processes. Identification of such factors as contaminant humics, optimum ore particle size for

biodegradation, microorganisms capable of biodegrading the humics present, the optimum physiochemical conditions for the culture and the removal of products will need to be made.

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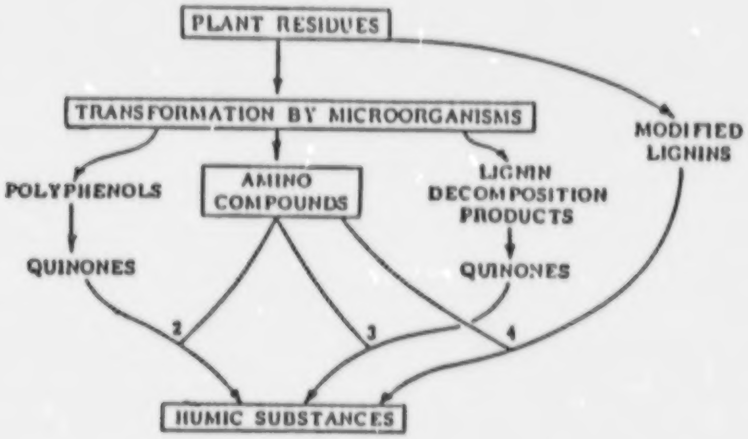
PLANT RESIDUES
TRANSFORMATION BY MICROORGANISMS
MODIFIED LIGNINS
POLYPHENOLS
AMINO COMPOUNDS
LIGNIN DECOMPOSITION PRODUCTS
QUINONES
QUINONES
2
3
4
HUMIC SUBSTANCES

Fig. 1 Mechanisms for the formation of soil humic substances. Amino compounds synthesized by microorganisms are seen to react with modified lignins reaction 4), quinones (reactions 2 and 3), and reducing sugars (reaction 1) to form complex dark-coloured polymers.

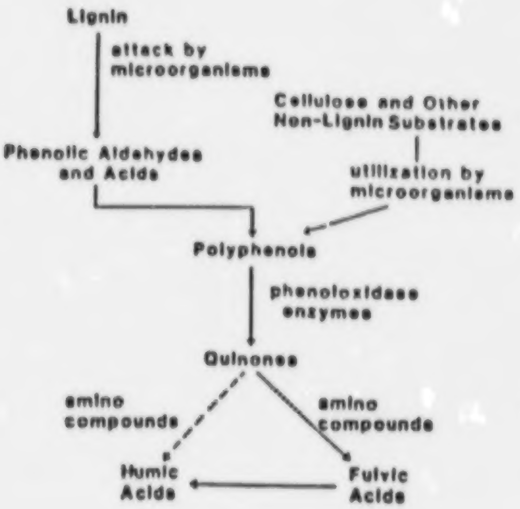
Lignin
attack by microorganisms
Cellulose and Other Non-Lignin Substrates
Phenolic Aldehydes and Acids
utilization by microorganisms
Polyphenols
phenoloxidase enzymes
Quinones
amino compounds
amino compounds
Humic Acids
Fulvic Acids

Fig. 2 Schematic representation of the polyphenol theory of humus formation.

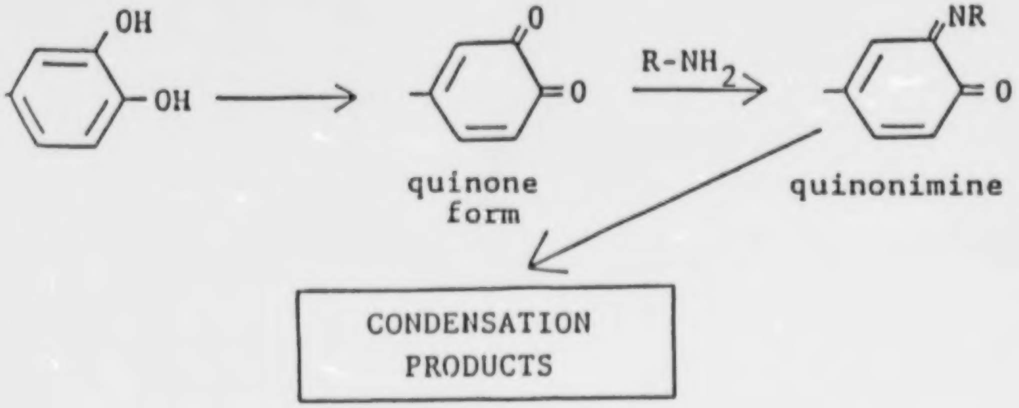
OH
OH
O
O
R-NH2
NR
O
quinone form
quinonimine
CONDENSATION PRODUCTS

Fig. 3 THE POLYPHENOL THEORY

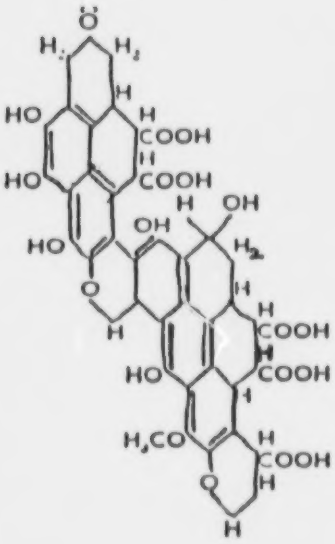
O
H2
H2
H
HO
H
COOH
H
HO
COOH
H
OH
OH
HO
H2
O
H
H
H
COOH
H
HO
COOH
H
H3CO
H
COOH
O
H
H

Fig. 4 A partial chemical structure of humic acid.

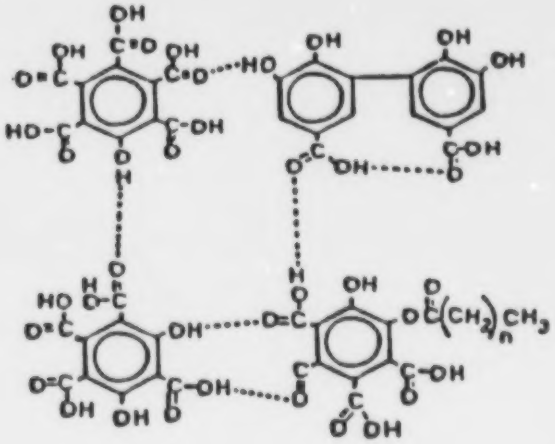

Fig. 5 A partial chemical structure of fulvic acid.

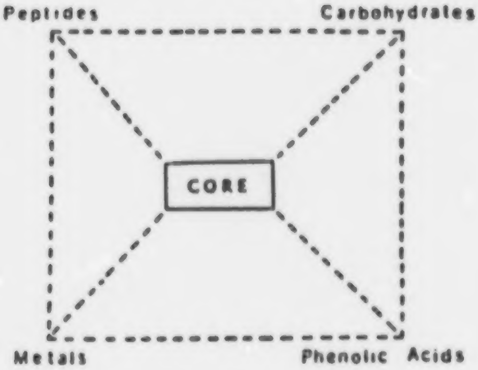
Peptides
Carbohydrates
CORE
Metals
Phenolic Acids

Fig. 6 Diagramatic representation of HA.

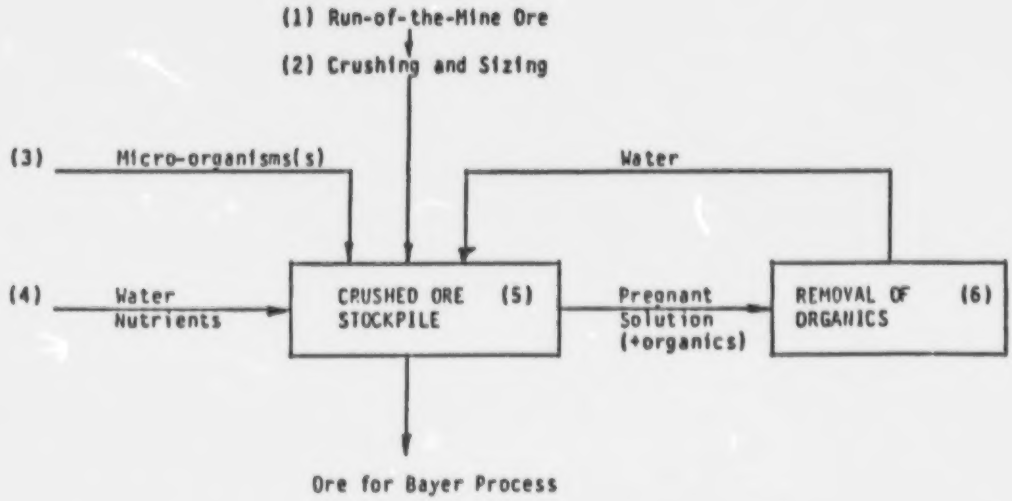
(1) Run-of-the-Mine Ore
(2) Crushing and Sizing
(3) Micro-organisms(s)
Water
(4) Water
Nutrients
CRUSHED ORE STOCKPILE (5)
Pregnant Solution (+organics)
REMOVAL OF ORGANICS (6)
Ore for Bayer Process

Fig. 7 Humic substances removal from ores by micro-organisms.

Table 1

Utilization of Humic and/or Fulvic Acids by Fund

Organism	Substrate	Incubation Period	% Degradation	Comments	Reference
Penicillium frequencies 1	HA (0.2%)	4 weeks	17.3	Czapek Dox broth.	Mathur & Paul 1967)
Penicillium frequencies 2	HA (0.2%)	4 weeks	17.3	Humic acids added as sole	Mathur & Paul 1967)
Aspergillus versicolor	HA (0.2%)	4 weeks	5.7	source of C and H.	Mathur & Paul 1967)
Fusarium sp.	HA (0.2%)	4 weeks	5.7	Photometric measrements.	Mathur & Paul 1967)
Grenalosporium sp.	HA (0.2%)	4 weeks	7.7		Mathur & Paul 1967)
Cunninghamella sp.	HA (0.2%)	4 weeks	7.7		Mathur & Paul 1967)
Panicillium 675	HA (0.2%)	4 weeks	7.7		Mathur & Paul 1967)
Penicillim frequentans	Ha (0.2%)	7 weeks	35.	Czapek Dox broth	Mathur & Paul 1967)
				HA as sole source of C & H	Mathur & Paul 1967)
				Replacement culture — shaken.	
				Photometric measurement.	
Mined soil population	FA (0.12)		ND	No degration-culture darken	
Poria subacida 17700	FA (0.05%)	24 days	45.3	Czapek Dox broth + Yeast Extr	Mathur (1969)
				Static culture. Photometric	
				Measurement (Loss of colour)	
Paria subacida 17780	FA (0.05%)	48 hours	66.7	As above except replacement	Mathur (1969)
				culture — no shaking.	
Splearia sp.	HA (0.12%)	8 weeks	33.3	Soil percolation column.	Burnes & Latter (1960)
Polystictus sp.	HA (0.12%)	8 weeks	42.8		Burnes & Latter (1960)
Barasmius preades	HA (0.05%)	1 weeks	51.	Czapek Dox broth. HA/FA as sole source of C & M.	Mathur (1960)
				Replacement/static culture.	
				Photometric measurment.	
Barasmius oreades	FA (0.05%)	1 weeks	18.	As above	Mathur (1970)
Fusarium solani	HA (0.1%)	40 days	40.	Mineral salts + C & M Suppl.	Khandelwal & Gaur (1980)
	(Manure)			Photometric measurement.	
Fusarium solani	HA (0.1%) (Soil)	40 days	18.	As above	Khandelwal & Gaur (1980)
Pen. roseopurpureum	HA (0.1%)	40 days	24.4	As above)	Khandelwal & Gaur (1980)
Asoergillus fumigatus	HA (0.1%)	40 days	31.1	As above)Manure HA	Knandelwal & Gaur (1980)

HA = Humic Acid FA = Fulvic Acid ND = Not Determined

Table 2

Utilisation of Humic and/or Fulvic Acids by Actinomycetles

Organism	Substrate	Incubation Period	% Degradation	Comments	Reference
Streptomyces antibioticus	HA (0.2%)	46 days	17.	Minerals salts + sand. Humic acid as sole source of C & n. Utilization measured by CO^2 evolution. Moisture – 60%	Monib *et al.* (1981)
S. noursei	HA (0.2%)	46 days	17.		
S. parvullus	HA (0.2%)	46 days	20.		
S. reticulus	HA (0.2%)	46 days	14.		
S. aureofaciens	HA (0.2%)	46 days	19.		
S. ramulosus	HA (0.2%)	46 days	15.		
S. qalilaeus	HA (0.2%)	46 days	11.		
S. albogrislolus	HA (0.2%)	46 days	16.		
S. viridogenes	HA (0.2%)	46 days	13.		
S. hygroscopicus	HA (0.2%)	46 days	14.		
S. venezulae	HA (0.2%)	46 days	13.		
S. olivaceus	HA (0.2%)	46 days	19.		
S. prasimus	HA (0.2%)	46 days	12.		
S. griseus	HA (0.2%)	46 days	17.		
S. griseoflavus	HA (0.2%)	46 days	17.		
S. viridochromogenes	HA (0.2%)	46 days	13.		
S. michiganensis	HA (0.2%)	46 days	15.		
S. rubrireticuli	HA (0.2%)	46 days	12.		
S. niveoruber	HA (0.2%)	46 days	11.		
S. phaeochromogenes	HA (0.2%)	46 days	12.		
S. fradiae	HA (0.2%)	46 days	12.		
S. purpurescens	HA (0.2%)	46 days	15.		
S. netropsis	HA (0.2%)	46 days	19.		
S. tendae	HA (0.2%)	46 days	12.		

Table 3

Utilisation of Humic and/or Fulvic Acids by Actinomycetles

Organism	Substrate	Incubation Period	% Degradation	Comments	Reference
Streptomyces violaceoniger	HA (0.2%)	46 days	15.	Mineral sals + sand.	Monib *et la.* (1981)
S. erythraeus	HA (0.2%)	46 days	17.	Humic acid as sole source of C & N.	Monib *et al.* (1981)
S. lavendulae	HA (0.2%)	46 days	21.	Utilization measured by CO^2	Monib *et al.* (1981)
S. fulvissimus	HA (0.2%)	46 days	17.	evolution	Monib *et al.* (1981)
Streptomyces sp. (A 31)	HA (0.1%)	40 days	20.0	Mineral salts supplemented	Khandelwal & Gaur (1980)
Streptomyces sp. (A 32)	HA (0.1%)	40 days	24.4	with C & N.	Khandelwal & Gaur (1980)
S. candidus (A 11)	HA (0.1%)	40 days	47.0	Tests using manure humic	Khandelwal & Gaur (1980)
S. candidus (A 12)	HA (0.1%)	40 days	44.4	acid.	Khandelwal & Gaur 1980)
S. garyphalus	HA (0.1%)	40 days	24.4	Photometric measurement.	Khandelwal & Gaur (1980)
S. rubescens	HA (0.1%)	40 days	28.9	(loss of colour).	Khandelwal & Gaur (1980)
S. violaceoruber (A 41)	HA (0.1%)	40 days	24.4		Khandelwal & Gaur (1980)
S. violaceoruber (A 42)	HA (0.1%)	40 days	31.1		Khandelwal & Gaur (1980)
S. candidus	HA (0.1%)	40 days	26.0	As above except soil HA.	Khandelwal & Gaur (1980)
Proactinomyces citreus	HA	150 days	ND	Decomposition of straw.	Ivanova (1979)
Proactinomyces corallinus	HA	150 days	ND		Ivanova (1979)
Micromonosporaceae family	HA	150 days	ND		Ivanova (1979)
Mixed actinomycetes (30)	HA	NS	ND	Czaoek Dox broth. HA sole	Mathur & Paul (1967)
Arthrobacter sp.	HA	4 weeks	11.5	source of C & N.	Mathur & Paul (1967)
Arthrobacter globiformis	FA	NS	ND	Stimulated by benzoate & lactate.	Rifai & Bertru (1980)

Table 4

Bacterial Utilisation Humic and/or Fulvic Acids

Organism	Substrate	Incubation Period	% Degradation	Comments	Reference
Anrobacterium 1	HA (0.2%)	7 weeks	11.5	HA — Sole source of C and/or H	Mathur & Paul 1967)
Agrobacterium 2	HA (0.2%)	7 weeks	7.5	(Czapek Dox broth)	Mathur & Paul (1967)
Pseudomonas sp.	HA (0.2%)	7 weeks	15.4	HA utilization measure by	Mathur & Paul (1967)
Bacillus sp.	HA (0.2%)	7 weeks	5.7	decrease in optical density.	Mathur & Paul (1967)
Mixed soil organisms	FA-1 (0.2%)	10 days	48.4	Soil culture — FA utilization	Kunc *et al.* (1976)
Mixed soil organisms	FA-2 (0.2%)	10 days	79.8	measured by CO_2release.	Kunc *et al.* (1976)
Pseudomonas sp. (V3)	FA-1 (0.2%)	6 hours	ND		Kunc *et al.* (1976)
Pseudomonas sp. (V3)	FA-1 (0.2%)	6 hours	ND	Pure cultures	Kunc *et al.* (1976)
Pseudomonas sp. (K 37)	FA-1 (0.2%)	6 hours	ND	FA utilisation followed	Kunc *et al.* (1976)
Pseudomonas sp. (K 37)	FA-1 (0.2%)	6 hours	ND	by CO_2 — release	Kunc *et al.* (1976)
Escherichia coli (K 12)	FA-1 (0.2%)	6 hours	ND		Kunc *et al.* (1976)
Escherichia coli (K 12)	FA-2 (0.2%)	6 hours	ND		Kunc *et al.* (1976)
Pseudomonas putida	FA		NA		Rifai & Dertru (1990)
Pseudomonas fluorescens	HA		NA		Gordienko & Kunz (1984)
Bacillus megaterium	HA		NA		Gordienko & Kunz (1984)
Nycobacterium sp.	HA		NA		Gordienko & Kunz (1984)

HA = Humic Acid: FA = Fulvic Acid: ND = No Degragation HA = Not available
FA-1 = lignofulvonic Acid FA-2 = Fulvenic Acid

MICROBIAL LEACHING OF GOLD FROM ORES AND MINERAL WASTES BY MEANS OF HETEROTROPHIC BACTERIA

by

STOYAN N. GROUDEV[1] *AND VENETA I. GROUDEVA*[2]

ABSTRACT

Different heterotrophic bacteria leached gold from two gold-bearing silicate ores and from a pyrite cinder in reactors with mechanical stirring. The leaching was carried out by bacteria growing in the presence of the mineral raw material being leached as well as by means of fermentation fluids obtained as a result of prior cultivation of the bacteria on glucose in the absence of mineral raw materials. The bacterial action was connected with the secretion of gold-oxidizing (peroxides) and gold-complexing (amino acids) agents. The leaching efficiency of bacterial fermentation fluids containing such metabolites was considerably increased by the addition of a chemical gold-oxidizing agent (potassium permanganate) and a disinfectant, sodium merthiolate (to prevent the growth of contaminating microoganisms). Over 80% of the gold was leached from the ores and the pyrite cinder within 2-3 days in reactors at 90°C by using such fermentation fluids.

Fermentation fluids of this type were also used to leach gold from the silicate ores in percolation columns. Respectively, 65.3 and 71.2% of the gold was leached from the two ores in this way.

The optimum leaching conditions for the different leaching processes were determined.

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[1] Research and Training Centre of Mineral Biotechnology, Higher Institute of Mining and Geology, Sofia 1156, Bulgaria.

[2] Department of Microbiology, Faculty of Biology, University of Sofia, Sofia 1421, Bulgaria.

LIXIVIATION MICROBIENNE DE L'OR CONTENU DANS DES MINERAIS ET DES RÉSIDUS MINÉRAUX PAR DES BACTÉRIES HÉTÉROTROPHES.

S.N. Groudev[1] *et V.I. Groudeva*[2]

RÉSUMÉ

Différents types de bactéries hétérotrophes peuvent extraire l'or contenu dans deux minerais siliceux et dans des cendres de pyrite, par brassage dans un réacteur. La lixiviation est assurée par des bactéries qui croissent en présence des minéraux bruts ou par la fermentation des fluides résultant de la culture de bactéries sur un milieu de glucose en l'absence de minéraux bruts. L'action bactérienne est liée à la sécrétion d'agents d'oxydation et de complexation de l'or (peroxydes et aminoacides). La lixiviation par les fluides de la fermentation bactérienne contenant ces métabolites est grandement améliorée lorsqu'on ajoute un agent oxydant de l'or (permanganate de potassium) et un désinfectant, le merthiolate de sodium (qui empêche la croissance de micro-organismes). Après 2 à 3 jours dans les réacteurs à une température de 90°C, plus de 80% de l'or a pu être extrait des minérais et des cendres de pyrite par ces fluides de fermentation.

D'autres fluides de fermentation du même type ont été utilisés pour extraire l'or des minerais siliceux dans des colonnes de percolation. On a réussi à extraire 65,3 et 71.2% d'or dans les deux minerais à l'aide de cette méthode.

On a aussi déterminé les conditions optimales pour les différents procédés de lixiviation.

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[1] Centre de Recherche et de Formation en Biotechnologie des minerais, Institut des Mines et de la Géologie, Sofia, Bulgarie

[2] Département de Microbiologie, Faculté de Biologie, Université de Sofia, Sofia, Bulgarie

INTRODUCTION

In the 1960's it was shown that different heterotrophic bacteria can dissolve gold from lateritic materials (Pares, 1964; Pares, 1965; Pares, 1968a; Pares, 1968b; Pares and Cuper, 1964; Pares and Martinet, 1964). These bacteria belong to well-known species and are widely distributed in soils and fresh waters. Most of these gold solubilizing bacterial species are members of the genus *Bacillus*. In all cases, however, the gold solubilization took place at low rates. The maximum concentration of gold in solution did not exceed 1.5 mg/L. The highest final gold extraction was 82% after 283 days of leaching.

More active gold solubilizing bacteria were isolated by the same research team from water and soil samples taken from gold deposits. These bacteria are related to the genus *Aeromonas*. The maximum gold concentration during leaching by the bacteria was as high as 10 mg/L. Gold was solubilized at slightly alkaline pH – from 8.0 to 8.5. The leach mechanism was found to be complex and no final conclusions were made.

More recently it was found that strains of well-known heterotrophic bacteria (*Bacillus megaterium*, *Bacillus mesentericus*, *Pseudomonas liquefaciens*, *Sarcina flava*, etc.) solubilized gold by producing gold oxidizing (peroxides) and complexing (amino acids, peptides, proteins and nucleic acids) agents (Korobushkina et al., 1974; Korobushkina et. al., 1983). The oxidizing agents turn the native gold into ionic state. The gold ions are then complexed by the different microbial metabolites. The efficacy of the process is dependent on the nature and levels of oxidizing and complexing agents in the culture medium. The dissolution is most active in the presence of aspartic acid, histidine and serine. However, the rates of gold solubilization and concentrations obtained are much lower than those of current industrial gold retrieval systems.

The gold solubilization is markedly accelerated when a chemical gold oxidant, e.g., sodium peroxide or potassium permanganate, is added to the culture fluids formed by different amino-acid-producing bacteria. The optimum pH for gold solubilization is from 9 to 10. Amino acid and protein fractions obtained from the culture fluids are also effective in gold solubilization. Thus, in presence of amino acid fractions, the concentration of gold was increased continuously and reached a maximum value of 64.4 mg/L in 360 days (Korobushkina *et al.*, 1983). In such long-term experiments colloid gold is also found together with dissolved gold.

Most data about the direct microbial solubilization of gold are obtained by experiments using dust gold and the shake-flask leaching technique under aseptic conditions. The data about microbial leaching of gold from ores and different mineral products are scarce (Polkin *et al.*, 1982, pp. 242-244; Groudev and Groudeva, 1988; Karavaiko and Vernikova, 1988). Due to the low gold content of these mineral raw materials, their leaching even under laboratory conditions is carried out by using relatively large samples (of several kilograms each) and appropriate facilities (percolation columns and reactors). Teaching under such conditions is similar to that which may occur under industrial conditions and which will not operate under aseptic conditions. It is imposible to keep such systems free from outside contamination. No information is available on the fate of the initial microbial populations as well as on the gold solubilization carried out by the mixed microbial populations established in these systems as a result of contamination during leaching. It is known that in some cases the leaching by means of culture solutions obtained as a result of prior cultivation of the relevant microoorganisms under aseptic conditions in the absence of minerals can be more efficient. It is possible to prevent the outside contamination of such solutions by supplementing them with an appropriate disinfectant.

This paper presents results from a study on microbial leaching of gold from two gold-bearing silicate ores and from a pyrite cinder.

MATERIALS AND METHODS

Three gold-solubilizing bacterial strains isolated from gold deposits and related to the species *Bacillus subtilis, Bacillus mesentericus and Bacillus sp.*, respectively, were used in the test work. Two mixed enrichment cultures of gold-solubilizing microorganisms were also used.

The pure strains and the mixed cultures were maintained in the nutrient medium suggested by Korobushkina *et al.* (1974). The medium was composed of 20.0 g NH_4Cl, 2.0 g KH_2PO_4, O.I. g $CaCl_2$, 0.5 g $MgSO_4$, 2.0 g NaCl, 50.0 g glucose in 1000 mL tap water. The pH was adjusted to 7.5.

The gold-bearing silicate ores used in this study contained 3.2 and 1.7 g/t Au, respectively. The gold was finely disseminated and the particle size was in the range of 10-30 microns. Quartz and feldspars were the basic minerals of the ores.

The pyrite cinder contained 2.3 g/t Au. Different iron oxides and quartz were the basic minerals of this sample. Its particular size was 90% minus 100 microns.

The microbial lelaching of the ores was carried out both in reactors and in percolation columns.

Ore samples crushed to minus 100 microns and varying in amount from 5 to 15 kg were leached in baffled cylindrical reactors with mechanical stirring. The density of the leach suspensions varied in the range of 10-50% and was adjusted to the desired level by addition of different amounts of leach solution. In some experiments, the above mentioned nutrient medium inoculated with a late-log-phase microbial culture was used as leach solution. The different inocula contained approximately 10^9 cells/mL each and were added in volume representing 10% of the total volume of the leach solution. The leaching was carried out at 35°C for a period of 14 days.

In the other experiments, microbial fermentation fluids obtained as a result of prior cultivation of the relevant microorganisms in fermenters in the absence of ore were used as leach solutions. Potassium permanganate was added to fermentation fluids at cocentrations varying from 1 to 15 g/L. A disinfectant, sodium merthiolate, was added in some experiments to prevent the growth of contaminating microorganisms. The leaching was carried out at temperatures varying in the range of 20 to 90°C for 5-14 days.

Column leaching of the ores was carried out in percolation PVC columns with 1800 mm effective length and 105 mm internal diameter. Each column was charged with 30 kg of ore crushed to minus 10 mm. Microbial fermentation fluids supplemented with potassium permanganate and sodium merthiolate were used as lealch solutions. The solutions were pumped to the top of the columns and percolated through the ore. Gold solubilization was monitored during the 150-day period at 30°C.

Freshly collected ore samples with their indigenous microbial populations were leached under otherwise similar conditions except that distilled water (with or without sodium merthiolate) or nutrient medium (with or without sodium merthiolate) were used as leach solutions.

The pyrite cinder was leached in reactors under the same conditions as used for the ores were.

To determine the cocentration of dissolved gold, samples of leach liquors were centrifuged for 10 minutes at 7500 x g to remove biomass and/or undissolved minerals. The supernatant was acidified to pH 1 with 10% wt/vol. hydrochloric acid and analyzed for gold by atomic absorption spectroscopy.

RESULTS AND DISCUSSION

The leaching of gold in reactors under non-aseptic conditions by means of microbial cultures growing on the organic substrate in the medium, i.e., on glucose, in the presence of the mineral raw material being leached was not efficient. Such systems were not selective in the production and maintainance of gold-solubilizing microorganisms. Mixed microbial populations were soon established as a result of outside contamination. Both the species composition and the cell number of the separate species varied in the different experiments. Most contaminants growing in the mixed cultures were members of the genera *Bacillus* and *Pseudomonas*. Various fungi were also present. The initially inoculated gold-solubilizing microorganisms were unfavourably affected by the contaminants. Some of the contaminants grew, depended on the amino acids secreted by the gold-solubilizing microorganisms. For that reason, the final gold extractions in the different experiments were low, mainly in the range of 2-5%. In some cases part of the solubilized gold was then precipitated as a result of decrease of pH of the leach solution caused by the secretion of organic acids by some contaminants.

The leaching of gold by means of fermentation fluids obtained as a result of prior cultivation of the relevant microorganisms on glucose in the absence of minerals and then supplemented with potassium permanganate and sodium fermentation fluids produced by the three pure microbial strains as well as by the two mixed enrichment cultures used in this study markedly differed from one another with respect to their gold-solubilizing activity. This was connected with the different composition of these fluids (Table 2). To achieve an efficient leaching, the total concentration of amino acids had to be higher than 5.0 g/L. The optimum concentration of potassium permanganate in leach solution was in the range of 2-5 g/L. The optimum leaching conditions regarding pH, temperature, pulp density and stirring rate were found to be 10-11, 90-100°C, 15-20% and 400- 600 rpm, respectively.

The amenability to leaching of the three mineral raw materials used in this study were different. Gold was more efficiently solubilized from the silicate ores than from the pyrite cinder. Under optimum conditions, over 80% of the gold was leached from the mineral raw materials in 2-3 days by using fermentation fluid formed by the most active microorganism used in this study — *B. subtilis* (Figure 1). However, the final gold extractions did not exceed 90% even after 10 days of leaching at 90°C. The maximum gold concentration in solution was as high as 1.1 mg/L. No precipitation of dissolved gold occured if the solution pH was maintained above 8.

Microbial fermentation fluids supplemented with potassium permanganate and sodium merthiolate leached gold efficiently from the ores in percolation columns (Figure 2). The results are similar to those obtained with the same ores but by means of cell-free fermentation fluids with similar chemical composition (Groudev and Groudeva, 1988), i.e., for achieving an efficient leaching it is not necessary to remove the microbial cells from the fermentation fluids. The optimum leaching conditions are summarized in Table 3.

Microbial leaching of gold with indigenous microorganisms of the gold-bearing ore was not efficient (Table 4). This was due not only to the fact that various contaminants grew in the leach systems but also to the fact that the indigenous microflora of the ores did not include microorganisms with an expressed gold-solubilizing activity. Most members of this microflora were related to the genera *Bacillus*, *Pseudomonas*, *Bacterium* and *Mycobacterium*. Various fungi and some autotrophic bacteria (thiobacilli and nitrifying bacteria) were also present. No specific gold-solubulizing microorganisms were isolated and probably such microorganisms don't exist. Each microbial culture (pure or mixed) producing both peroxides and gold-complexing amino acids can leach exposed gold at alkaline pH. However, most microorganisms producing gold-complexing amino acids are poor producers of peroxides. For that reason, the supplementation of the amino acid-containing fermentation fluids with a gold-oxidizing agent, e.g., potassium permanganate, is absolutely necessary for achieving an efficient gold solubilization.

It has been shown that protein hydrolysates obtained after alkaline hydrolysis of rich-in-protein substances, e.g., mycelium of the fungus *Aspergillus niger*, waste products from agricultural processes and food industries, etc., are also effective in gold solubilization in the presence of gold oxidants (Polkin *et al.*, 1982; Karavailo and Vernikova, 1988) and their utilization is more attractive from an economic point of view than some of the species used in the work reported here.

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TABLE 1

Leaching of Gold from Mineral Raw Materials in Reactors by Means of Microobial Fermentation Fluids Supplemented with Potassium Permanganate

Microorganism	Ore No. 1	Ore No. 2	Pyrite cinder
	Gold extraction in 10 days (%)		
Bacillus subtilis	80.6	88.0	70.4
Bacillus mesentericus	28.0	31.5	23.4
Bacillus sp.	57.2	60.2	44.4
Mixed enrichment culture 1	20.8	24.4	19.8
Mixed enrichment culture 2	51.4	40.4	37.8

The leaching was carried out at 35°C in the presence of sodium merthiolate

TABLE 2

Amino acid concentration (in % from the total concentration of amino acids) in fermentation fluids formed by the gold- solubilizing bacteria used in this study

Amino acid	Microorganism		
	Bacillus subtilis	Bacillus mesentericus	Bacillus sp.
Alanine	12.0	7.1	9.1
Aspartic acid	18.9	16.0	21.3
Glutamic acid	16.9	16.5	13.2
Glycine	3.7	7.2	1.2
Histidine	9.2	10.8	15.5
Lysine	-	2.6	-
Methionine	9.0	5.0	10.5
Phenylalanine	5.9	4.1	5.1
Serine	14.0	11.9	14.5
Threonine	-	2.5	-
Tyrosine	-	1.5	-
Valine	-	1.9	-
Total Concentration of amino acids (g/L)	8.2	5.3	8.0

TABLE 3

Optimum leach parameters in the column leaching of the gold- bearing ore samples

Variable	Ore No. 1	Ore No. 2
Leach solution composition: Fermentation fluid obtained after cultivation of Bacillus subtilis and containing amino acids (total), g/L	>5.0	>5.0
Supplements to the fermentation fluid: Potassium permanganate, g/L	5.0 to 10	5.0 to 7.0
Sodium merthiolate, g/L	1.0	1.0
pH	10.5	11.0
Eh, mV	>500	>500
Temperature, °C	30	30
Irrigation rate, 1/ton.day	100 to 140	90 to 120
Irrigation – rest periods, ratio in days	3.2 : 1	2.1 : 1
Length of the use of leach solution until its replacement by a new batch of fresh solution, days	7 to 15	10 to 15
Gold concentration in the pregnant leach solution, mg/L	0.1 – 0.5	0.06 – 0.3

TABLE 4

Column leaching of fresh gold-bearing ores with native microbial population

Leach solution	Ore No. 1	Ore No.2
	Gold extraction in 150 days (%)	
Nutrient medium	4.5	5.4
Nutrient medium with sodium merthiolate	0.7	0.8
Distilled water	0.8	1.0
Distilled water with sodium merthiolate	0.3	0.5

The nutrient medium specified by Korobushkina *et al.* (1974) was used.

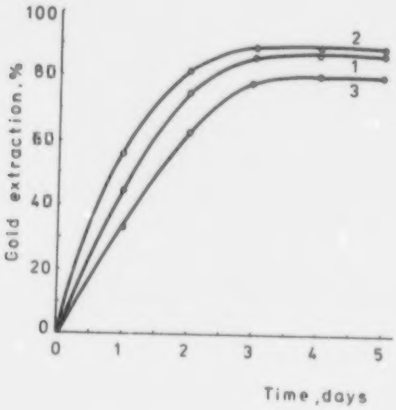

Fig. 1 Leaching of gold from mineral raw materials in reactors at 90°C by means of fermentation fluids produced by *Bacillus* subtilis and supplemented with potassium permanganate

1 – ore No. 1

2 – ore No. 2

3 – pyrite cinder

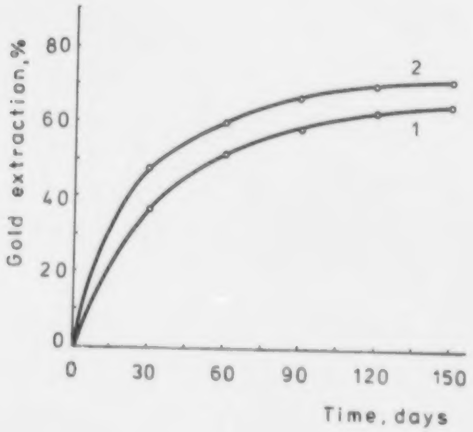

Fig. 2 Leaching of gold-bearing silicate ores in columns by means of fermentation fluids produced by *Bacillus subtilis* and supplemented with potassium permanganate

1 – ore No. 1

2 – ore No. 2

BIOLOGICAL TREATMENT OF SILVER-CONTAINING ORES AND WASTES

by

L.L. LUBAVINA[1], *N.N. LYALIKOVA*[2], *P.M. SOLOZENKIN*[1], *N.N. BUJANOVA*[1]

ABSTRACT

A combined technological scheme of processing of difficult-to-treat low grade silver ore was proposed. The ores and wastes were treated with bacterial strain of *Bacillus mucilaginosus*, then flotation under usual reagent regime was carried out.

As a result the silver extraction in the concentrate was enhanced by 5-25%.

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[1] Institute of chemistry Tadzhik Academy of Sciences, Dushanbe.

[2] Institute of Microbiology Academy of Sciences, Moscow.

TRAITEMENT BIOLOGIQUE DE MINERAIS ARGENTIF RES ET DE RÉSIDUS.

*L.L. Lyubavina**, N.N. Lyalikova***, *P.M. Solozenkin**, *N.N. Buyanova**.

RÉSUMÉ

Un procédé technologique combiné pour le traitement de minerai à faible teneur en argent est proposé. Les minerais et les dechets sont d'abord traités par *Bacillus mucilaginous* et ensuite par flottation à régime d'agitation standard. Une augmentation de l'extraction de l'argent contenu dans le concentré, de l'ordre de 5 à 25%, a été obtenu.

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* Institut de Chimie, Académie des Sciences du Tadjikistan, Douchanbe,

** Département de Microbiologie, Académie des Sciences de l'URSS, Moscou

INTRODUCTION

The extraction of associated metals from ores can be one of the ways to cope with raw material shortages.

Nowadays the techniques of hydrometallurgy and biohydrometallurgy are used for extraction of noble metals. Such non-ferrous metal ores as copper, copper-bismuth, lead-zinc and other polymetallics are the main source of silver. Those ores give 80% of the world silver production.

Silver extraction from silicate-based ores is very complicated. We've studied the possibility of silver extraction from two specimen of low grade silver ore, containing 70-75% of silicon oxide and 10-15% of alumina. More than 30% of silver in ore is in the form of emulsion impregnation in quartz, which makes its extraction more difficult. Rather often silver is covered with the sericite-chloryte jacket. Presence of the sludging alumina is a cause of difficulties also. A part of silver is contained in the lead and zinc sulfides and is transferred into the concentrate according to th floating ability of these sulfides. The sieve analysis of the ore has shown that 60-75% of the valuable components were present in the large classes.

These peculiarities of silver distribution allowed us to propose a combined technological scheme of processing of the ores under consideration. The attempt was made to release silver from silicates by means of biotreatment, which was suppose to improve silvers floating ability. For this aim Bacillus mucilaginosus culture was chosen. This species is close to *B. polymyxa* and *B. mucilaginosus* grows on synthetic media with carbohydrates. Ammonium salts or urea are sources of nitrogen. Sucrose can be substituted for malassa.

The ore was preliminary crushed so that 70% of particles belong to 0,063 mm class. After bacterial treatment the sulfidization f oxidized minerals with sodium sulfide (Na_2S-400 g/t) was carried out. The following flotation was performed under usual reagent regime:butyl xanthagenate — 65 g/t (the main flotation) + 35 (control flotation), frother T-80 100 + 100 g/t (main and control flotation).

At the beginning of the research work the bacteria was grown in the ore presence for 1-5 days. Formation of bacterial slimes and silicons gel was observed in the case of such biotreatment, which impeded the flotation process. Before flotation it was necessary to wash the ore with 0.1% alkali solution and then with water until washings became neutral.

The results of flotation carried out after bacterial treatment of dressing mill wastes by B. mucilaginosus culture, are given in Table 1. In the most successful experiments we've managed to extract in addition about 72% of silver from these wastes, containing also lead and zinc. Recovery of these metals in the concentrate after bacterial treatment was increased by 20-30% as well.

In subsequent experiments the preliminary grown culture was used. The ore biotreatment has been carried out during 6-18 hours on the rotary shaker. The ore and cultural fluid ratio is of great significance. As follows from Table 2, the best silver extraction takes place at the pulp density of S:L = 1:50.

The results of bacterial pretreatment of the second ore specimen are given in Table 3. One can see the same regularity in relation to the pulp density. As follows from the table, the silicon amount transferred into solution increases in the case of more diluted pulp.

Probably the silver flotation ability increases owing to the release of silver because of the biological alteration of the silicate core. Some of the bacterial metabolites can be acting as flotation reagent.

These results show that the biological treatment enhances the silver extraction from wastes and hard-dressing ores by 5-15%.

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TABLE 1

Influence of 48 hours bacterial treatment on silver recovery from tailings of sulphide flotation

Material	Experimental condition	Silver recovery, %
Concentrate	Bacterial treatment	66.5
Tailings		33.5
Concentrate	Bacterial treatment	68.7
Tailings		31.3
Concentrate	Blank, treatment with sterile media	44.8
Tailings		55.2

TABLE 2

Dependence of Silver Recovery on Bacterial Pretreatment Conditions

Material	Conditions of bacterial pretreatment (S:L)	Recovery of silver, %
Concentrate	1:10	45.9
Tailings		54.1
Concentrate	1:25	49.8
Tailings		50.2
Concentrate	1:50	61.6
Tailings		38.4
Concentrate	Blank, without bacterial pretreatment	44.6
Tailings		55.4

TABLE 3

Recovery of silver in result of flotation of low grade lead-zinc ore after bacterial pretreatment

Material	Conditions of bacterial pretreatment (S:L)	Concentration of silicon in sol. SiO_2 (g/L)	Content of silver g/t	Recovery of Silver, %
Concentrate	1:10	0.11	278.2	49.9
Tailings			28.9	50
Concentrate	1.25	0.19	310.1	61.6
Tailings			23.2	38.4
Concentrate	1:50	0.28	468.5	72.0
Tailings			10.1	27.9
Concentrate	Blank, without bacterial pretreatment	0.06	152.8	45.9
Tailings			34.1	54.1

WASTE TREATMENT

The Use of Immobilised Biomass For The Continuous Recovery of Uranium From Elliot Lake Ore. First Pilot Plant Results.

M. Tsezos[1], *R.G.L. McCready*[2], *J. Salley*[2], *J.P. Cuif*[3].

ABSTRACT

A proprietary process for the immobilization of microbial biomass into particles with specified technical characteristics has been developed by Dr. Tsezos at McMaster university. This process was used to immobilize inactive *Rhizopus arrhizus* biomass which has been shown to be an excellent uranium biosorption agent. The immobilized *Rhizopus arrhizus* biomass was tested for its potential for the continuous recovery of uranium from the industrial bioleach solutions that are presently produced by Denison Mines in the Elliot Lake area of Canada. At first a series of ten complete uranium adsorption-elution and kinetic batch tests were carried out at pH 4 settled mine bioleachate. Subsequently a laboratory scale continuous pilot plant incorporating an upflow packed bed uranium biosorption reactor was operated successfully on the same mine bioleachate for 14 successive uranium adsorption-elution cycles at McMaster University. The encouraging results led to the design, construction and operation of a larger scale (30x) pilot plant also charged with similar immobilized biomass which due to the space , facilities and logistic demands was constructed and started operating at the CANMET laboratories in Ottawa. The paper will present a summary of the results and the progress made by CANMET and McMaster University in the development of this novel biohydrometallurgical process, which following almost a decade of study, appears to be very promising.

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[1] McMaster University, Chemical Engineering, Hamilton, Ontario, Canada

[2] Biotechnology Section, CANMET, Ottawa, Ontario, Canada

[3] Visiting scientist at CANMET, Ottawa, Ontario, Canada

UTILISATION DE LA BIOMASSE IMMOBILISÉE POUR LA RÉCUPÉRATION CONTINUE DE L'URANIUM DES PRODUITS DE LIXIVIATION DU MINERAI D'ELLIOT LAKE. RÉSULTATS OBTENUS DE LA PREMIÈRE USINE PILOTE

M. Tsezos[1], *R.G.L. McCready*[2], *J. Salley*[2], *et J.P. Culf*[3]

RÉSUMÉ

Un procédé protégé d'immobilisation de la biomasse microbienne dans des particules ayant des caractéristiques techniques spécifiques a été mis au point par Dr. Marios Tsezos de l'université McMaster. On a utilisé ce procédé pour immobiliser la biomasse inactive de *Rhizopus arrhizus*, qui s'est révélé être un excellent agent de biosorption de l'uranium. La biomasse immobilisée de *R. arrhizus* a fait l'objet d'essais visant à déterminer son potentiel pour la récupération continue de l'uranium contenu dans les produits de lixiviation de Dennison Mines dans la région d'Elliot Lake, au Canada. On a d'abord procédé à une série de dix essais d'adsorption-élution et à des essais cinétiques en vrac sur des produits de lixiviation de pH4. On a ensuite traité les mêmes produits de lixiviation dans une installation de laboratoire pilote, munie d'un réacteur de biosorption d'uranium, à lit compact, fonctionnant par flux ascendant. Les résultats ayant été encourageants, on a construit et mis en service une installation pilote de plus grande capacité (30 fois plus) pour le traitement des mêmes produits. En raison des contraintes d'espace et de logistique, et pour plus de facilité, l'installation a été construite dans les laboratoires de CANMET à Ottawa. Cet article présente un résumé des résultats obtenus et des progrès accomplis par CANMET et l'université McMaster dans le développement de ce procédé biohydrométallurgique qui, après presque dix ans de recherche, semble très prometteur.

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[1] Université McMaster, Département de Génie Chimique Hamilton (Ontario) Canada

[2] Section Biotechnologie, CANMET, Département d'Énergie, Mines et Ressources Ottawa (Ontario) Canada

[3] Scientifique invité à CANMET, Ottawa, (Ont.) Canada

Introduction:

Over the past decade the detailed study of the biosorption of uranium by inactive microbial biomass has shown that the biomass of *Rhizopus arrhizus* is an excellent selective adsorbent for the extraction and recovery of uranium from dilute and complex aqueous solutions.[1,2,3]

The inherently small particle size of the microbial biomass, however poses significant problems in applying biosorption as an industrial process The efficient and effective separation of the loaded microbial biomass from the barren solution following biosorption is difficult. The immobilization of the microbial biomass into particles of desirable mechanical and physical-chemical properties has been suggested as the answer to the problem.

A proprietary process for the immobilization of any microbial biomass into particles with a low (less than 15% w/w) content of inactive additives, high mass transfer rates and custom specified other technical characteristics has been developed by M. Tsezos at McMaster University.[4] This process has been used to successfully immobilize inactive *R.arrhizus* microbial biomass which was grown under optimized growth conditions and has been shown to be an excellent selective uranium adsorbent.[3]

This immobilized *R arrhizus* biomass was tested for its potential for the continuous selective extraction and recovery of uranium from Denison Mines bioleaching process water from the Elliot Lake district of Canada.

Results and Discussion

The testing procedure started with a series of ten consecutive batch equilibrium and kinetic biosorption-elution tests carried out at pH = 4 using filtered bioleachate. The detailed experimental results from the batch uptake tests have shown that uranium is biosorbed at a satisfactory rate and after an initial drop in the uranium equilibrium uptake capacity, the biosorbent particles exhibit a robust uranium uptake capacity of about 40 mg/g.[3] The prolonged agitation of the same immobilized biomass particles during the batch tests (over 100 days) led to the apparent reduction of the immobilized biomass uranium uptake capacity (Figure 1).

In order to further explore, the immobilized biomass process application potential and minimize attrition losses it was decided to construct and operate a laboratory scale continuous pilot plant using a 12 inch long upflow packed bed reactor. The pilot plant was operated at McMaster University for 12 consecutive continuous uranium biosorption/ elution cycles. Uranium was loaded on the immobilized biomass from pH = 4, filtered bioleachate which contained uranium in the range of 80 mg/l to 250 mg/l, iron and a large array of other cations and anions. Consistent breakthrough curves were observed for the 12 runs as illustrated on Figure 2.[3] The pilot plant produced concentrated uranium carbonate eluate solutions with concentrations exceeding 5000 mg/l uranium.[3] Highly favourable elution curves were also observed (Figure 3).[1] Detailed description of the results from the above work has been reported to literature[3].

The successful operation of the small pilot plant led to the design construction and operation of a second scaled up uranium biosorption pilot plant which makes use of a 6 feet long upflow packed bed reactor. Because of the logistic difficulties associated with its operation, the larger pilot plant was constructed and operated at the biotechnology laboratories of CANMET in Ottawa, Ontario, Canada. This second pilot plant was also operated on pH = 4, settled, Denison Mines bioleachate. Results available from the first 4 uranium biosorption/elution cycles have confirmed results of the first mini pilot plant. Average biomass Uranium uptake capacities of close to 40 mg/g and complete uranium elution with elute uranium concentrations of 12,000 mg/l have been observed. Figure 4 shows the uranium breakthrough curve observed during the fourth cycle of the larger pilot plant operation. The corresponding uranium elution curve is shown on Figure 5 where a peak uranium elution concentration of close to 10,000 mg/L is observed. Another important characteristic of the elution curve is the sharp, well defined and tight shape of the curve which signifies that elution is completed with a small volume of eluent.[2,5] Mass balances on the uranium retained and subsequently eluted from the immobilized biomass column suggest that the recovery of the biosorbed uranium is complete.

Following the first uranium biosorption/elution cycle the observed packed bed average uranium uptake capacity dropped from 60 mg/g to about 39 mg/g. Similar reduction in the immobilized biomass uranium uptake capacity was also observed during the operation of the first smaller, pilot plant. Extensive work on the study of this reduction in uptake capacity in the course of the last year at McMaster University has suggested aluminum ions as responsible for a competing co-ion effect. Detailed results on the mechanism of aluminum interference will be reported soon in the literature. The operation of the pilot plant will continue in order to test the endurance of the immobilized biomass and its response to long regular use. At the same time discussions on the installation of an industrial scale pilot plant have started with Denison Mines.

Conclusions

It has been demonstrated that a proprietary process for immobilizing biomass can produce biosorbent particles that can operate like conventional ion exchange resins in a continuous mode for the extraction and recovery of metal values from industrial complex, weak solutions.

The uranium case study has shown that uranium can be successfully extracted and fully recovered from the complex bioleach solutions produced by Denison Mines in the Elliot Lake area of Canada.

The present work reconfirmed the industrial application potential of biosorption as a process for the selective extraction of metals from complex, weak solutions.

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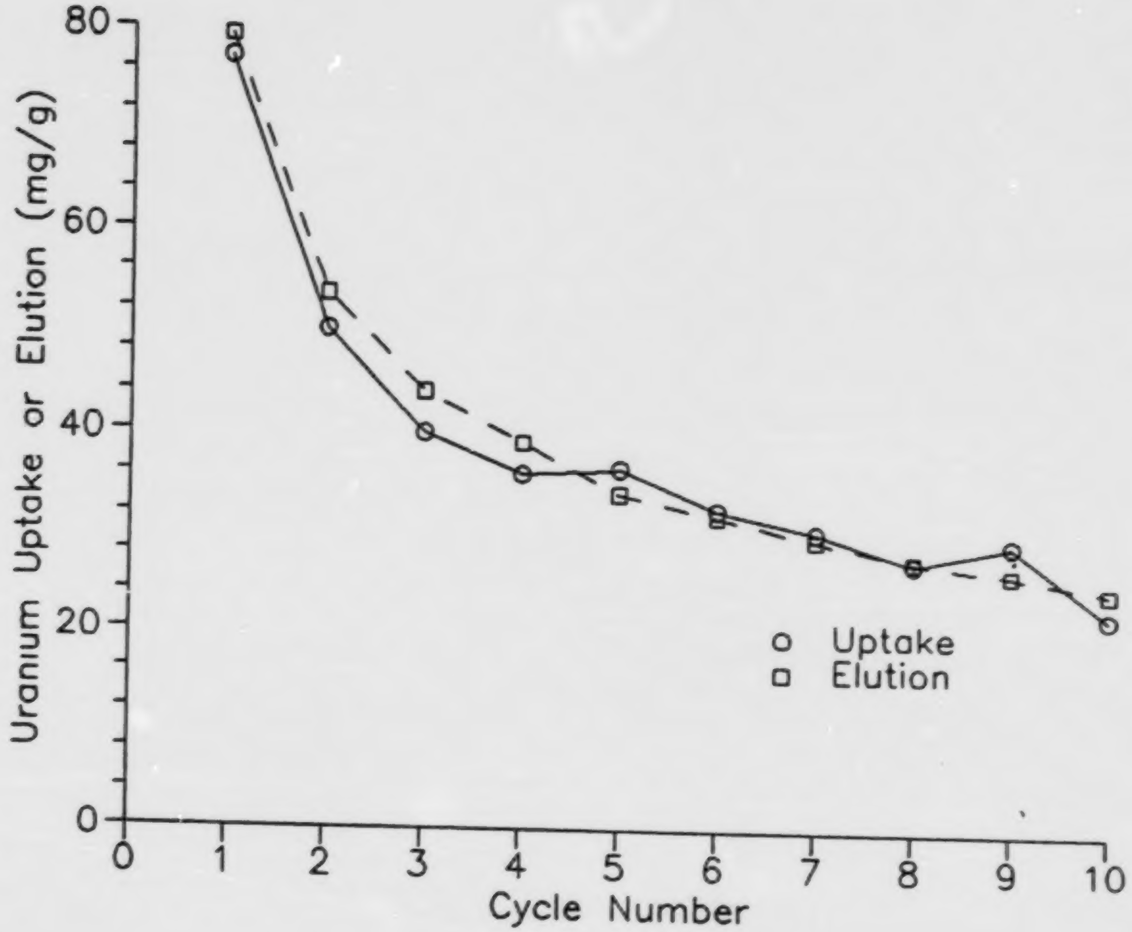

Fig. 1 Batch equilibrium uranium uptake results, immobilized *R. arrhizus* pH = 4, 20 C.

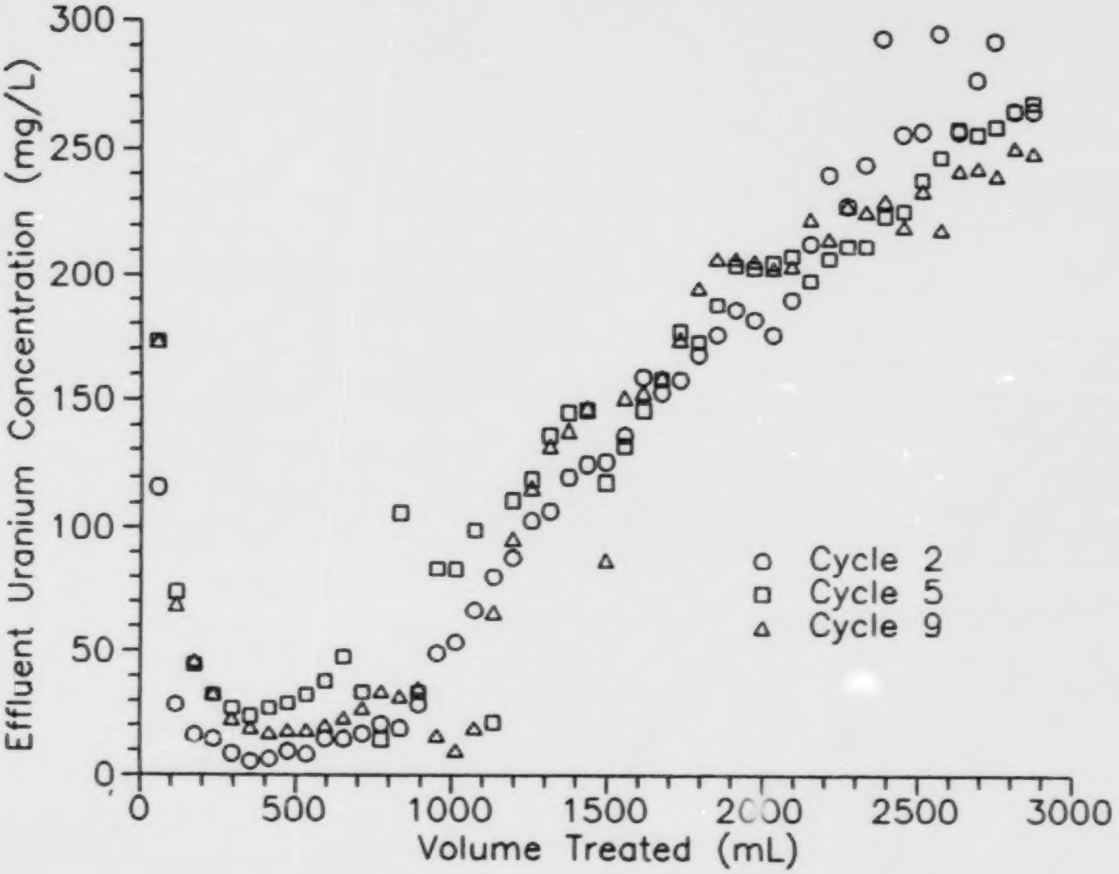

Fig. 2 Uranium uptake breakthrough curves from the first pilot plant, pH = 4.

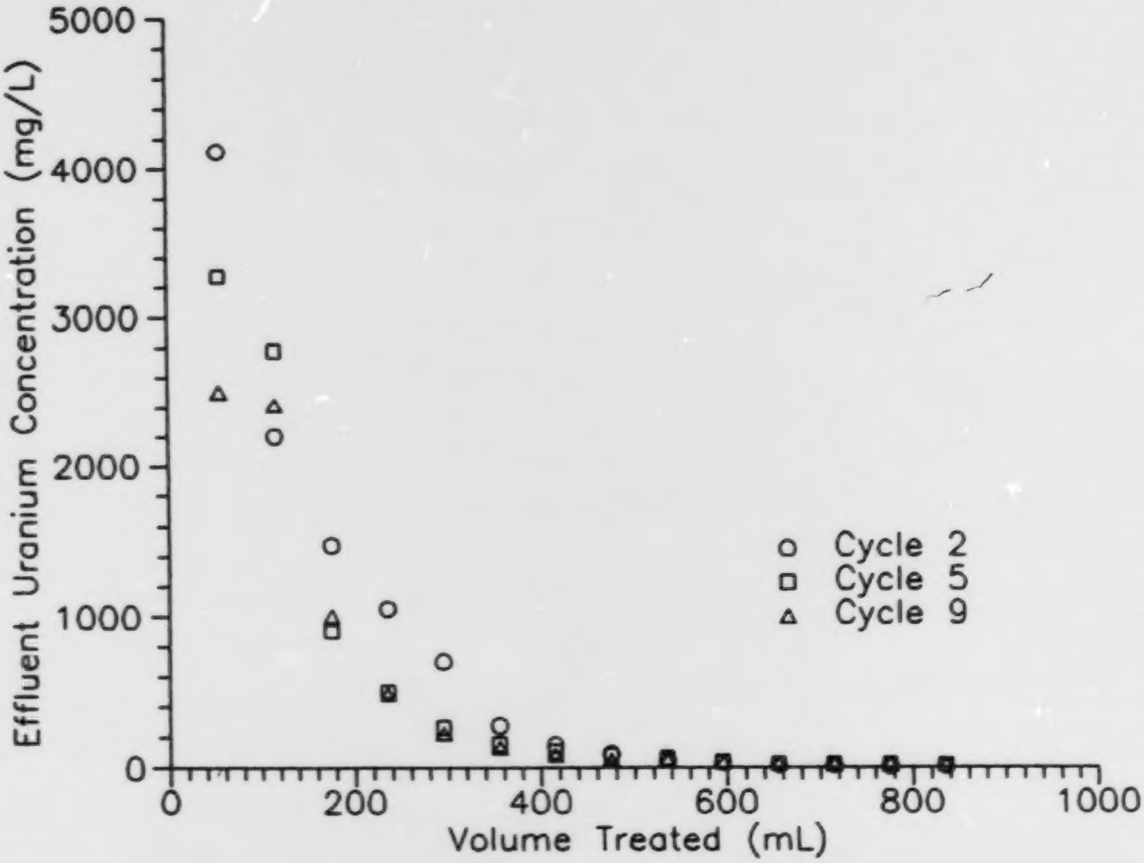

Fig. 3 Uranium elution curves from the first pilot plant.

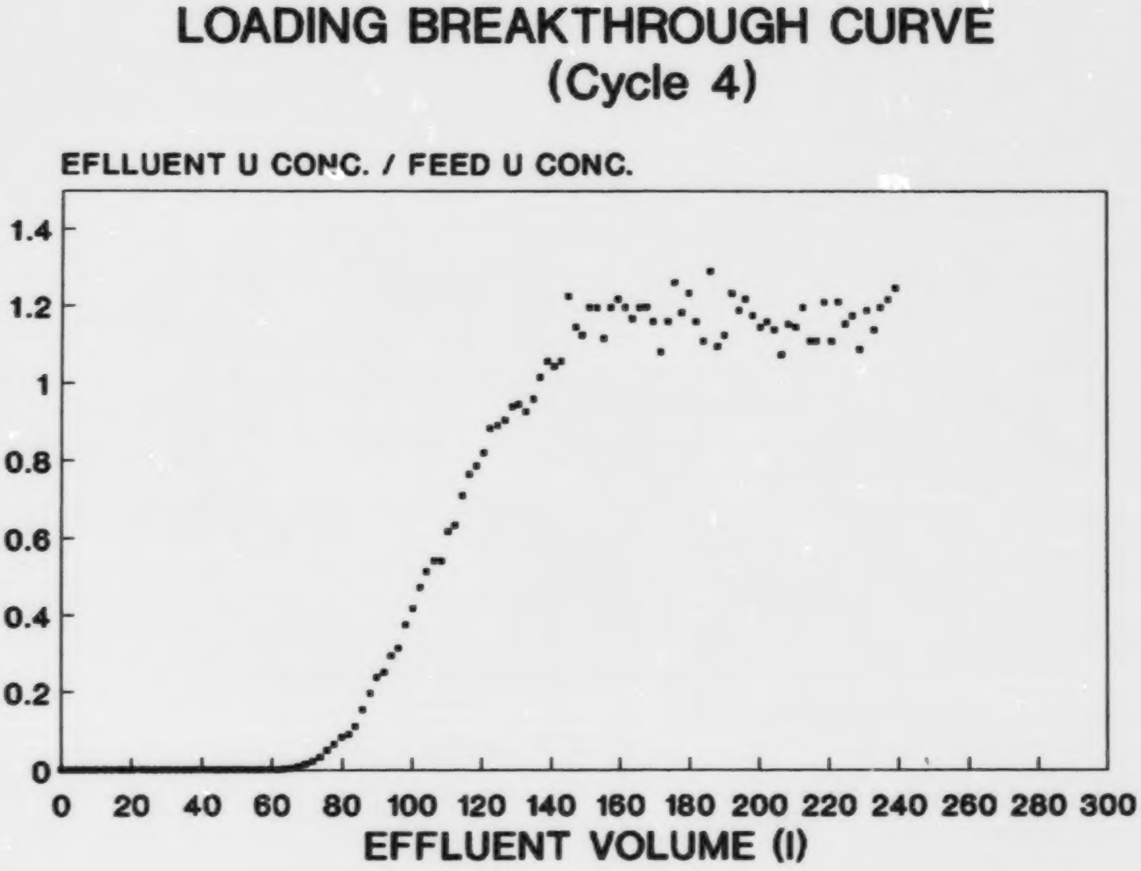

Fig. 4 Uranium breakthrough curve observed at the fourth cycle of the larger pilot plant, pH = 4.

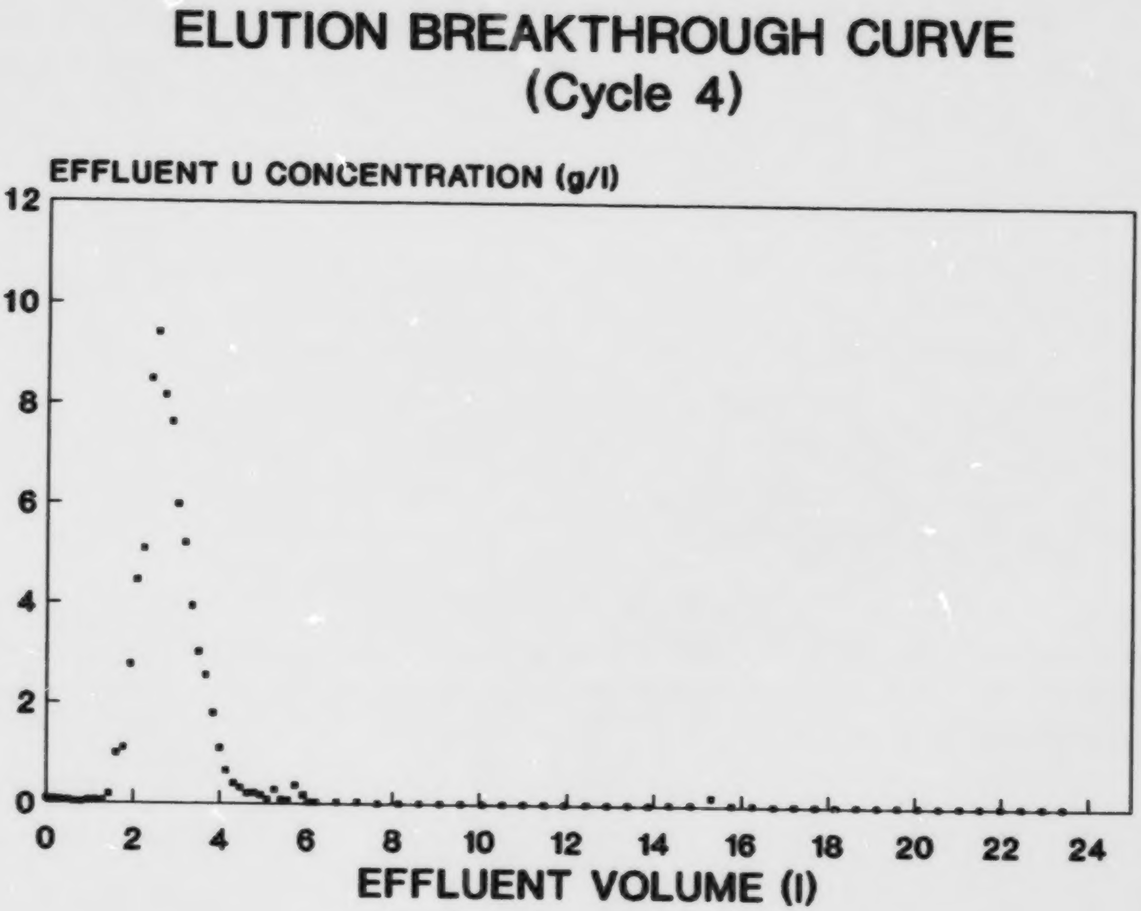

Fig. 5 Uranium elution curve from the fourth cycle of the larger pilot plant.

BIOLOGICAL DEGRADATION OF CYANIDE IN COMPLEX INDUSTRIAL WASTEWATERS

by

G.M. WONG-CHONG Baker/TSA, inc.

ABSTRACT

Biological degradation of cyanide in a complex industrial wastewater was examined; the wastewater contained: 100-250 mg/L ammonia, 72-182 mg/L cyanide, organics (1100 mg/L phenols), 500-600 mg/L thiocyanate. Activated sludge treatment of this wastewater was examined in batch and continuous flow reactor tests using synthetic and real wastewaters; all tests produced cyanide treatment of <0.1 mg/L. Batch reactor tests showed cyanide gradation was a first order reaction (i.e., $dC/dt = -kC$ where C = cyanide concentration), and cyanide inhibited both ammonia oxidation (nitrification) and thiocyanate degradation at levels above 0.5 and 2.0 mg/L respectively. Cyanide degradation in the continuous flow reactor tests can be described by a reaction rate $k = 1/HRT\ [Co/C-1]$ where HRT = reactor hydraulic residence time, and a specific reaction rate $K - k/MLVS$ where MLVS = reactor mixed liquor volatile solids concentration. Specific reaction rates for three series of tests were: a-synthetic wastewater without thiocyanate, $K = 0.245\ (mg/L \cdot day)^{-1}$, b) synthetic wastewater with thiocyanate, $K = 0.090\ (mg/L \cdot day)^{-1}$ and c) actual wastewater, $K = 0.049\ (mg/L \cdot day)^{-1}$. These differences in specific reaction rates indicate that thiocyanate and other materials in the actual wastewater inhibited cyanide degradation.

DÉGRADATION BIOLOGIQUE DU CYANURE DES EAUX USÉES INDUSTRIELLES

G. M. Wong-Chong, Baker/TSA, inc.

RÉSUMÉ

La dégradation biologique du cyanure des eaux usées industrielles a été étudiée. Les eaux usées contiennent : 100-250 mg/L d'ammoniac, 72-182 mg/L de cyanure, des matières organiques (1-100 mg/L phénols), 500-600 mg/L de thiocyanate. Le traitement d'eaux usées véritables et artificielles par les boues activées a été étudié dans le cas de réacteurs à débit continu et intermittent; pour tous les essais, la concentration finale du cyanure était inférieure à $<0{,}1$ mg/L. Les essais dans les réacteurs à débit intermittent montrent que la dégradation du cyanure est une réaction de premier ordre (c.-à-d. $dC/dt = -kC$, où C = concentration de cyanure) et que le cyanure inhibe l'oxydation de l'ammoniac (nitrification) et la dégradation du thiocyanate à des concentrations supérieures à 0,5 et 3,0 mg/L respectivement. La dégradation du cyanure dans les réacteurs à débit continu peut être décrite par l'équation de la vitesse de réaction suivante k-1/HRT[Co/C-1], où HRT = temps de séjour dans le réacteur hydraulique, et par la vitesse spécifique de réaction K-k/MLVS où MLVS = concentration des solides volatils dans les liqueurs agitées. Les vitesses de réaction spécifiques pour trois séries de tests sont les suivantes : a) eaux usées artificielles sans thiocyanate, K = 0,245 (mg/L.jour)-1, b) eaux usées avec thiocyanate, K = 0,090 (mg/L.jour)-1, c) eaux usées véritables, K = 0,049 (mg/L.jour)-1. Les différences entre les vitesses de réaction spécifiques indiquent que le thiocyanate et les autres substances présentes dans les eaux usées véritables inhibent la dégradation du cyanure.

INTRODUCTION

Many industrial process wastewaters contain complex mixtures of compounds of environmental concern, including cyanide. Examples of such wastewaters are those from coal coking, coal gasification and petroleum refining. These industrial wastewaters can contain ammonia, cyanide, phenols and thiocyanate, and discharge of these wastewaters into receiving streams requires treatment to meet stringent effluent quality limits. As an example, Table 1 compares typical wastewater composition from a coke plant and the discharge effluent quality which must be attained.

Compliance with the discharge effluent limits can be achieved by biological treatment. However, design and operation of the biological treatment process require an understanding of the various microbial processes/reactions which contribute to the overall treatment of these complex wastewaters. This paper reports on a study which was conducted to understand the fate and effects of cyanide on the biological (activated sludge) treatment of coke plant wastewaters. Specific objectives of the investigation were to determine:

- Fate of cyanide in the course of bio-treatment: biodegradation or stripping;
- The order of the degradation reaction, and reaction rate;
- Effects of other wastewater constituents on cyanide degradation rate; and
- Effect of cyanide on the degradation of other wastewater constituents.

MATERIALS AND METHODS

The study examined activated sludge treatments of synthetic and actual coke plant wastewater in both batch and continuous flow reactor systems. Synthetic wastewater was formulated according to the composition shown in Table 2; essential elements being ammonia, cyanide, phenol, thiocyanate (the concentrations of which were varied or omitted as desired) and sodium chloride (coke plant wastewaters contain high concentrations of chloride and dissolved solids).

The coke plant wastewater used was pretreated by steam stripping to reduce its ammonia concentration to about 75 to 150 mg/L. This wastewater was amended with ammonia, cyanide, phenol and thiocyanide to concentrations as desired. Also phosphorus either as the acid or as ammonia phosphate was added as a nutrient supplement.

The microbial sludge used was developed from seed material collected from a biological treatment plant which processed coke plant wastewaters. The sludge was acclimated/developed to contain an aggressive nitrifying population.

A typical batch experiment entailed the following:

- Mix an aliquot of wastewater with an aliquot of acclimated sludge to achieve desired sludge and chemical concentrations.
- Keep sludge/wastewater mixture agitated and aerated.
- Monitor constituent(s) of concern with the passage of time by periodically withdrawing samples for analysis.

A typical batch experiment involved about 4 to 8 liters of sludge/wastewater mixture and lasted from 6 to 24 hours.

Continuous flow experiments were conducted in completely mixed reactors fitted with integral clarification within the reactor (see Figure 1). These continuous flow reactors were operated at ambient temperatures (20 to 25°C) and mixed liquor dissolved oxygen concentrations were maintained at about

1 to 2 mg/L. The dissolved oxygen concentration in the reactor could be changed by adjusting either air flow rate and/or mixer speed. Each continuous flow reactor experiment lasted from one to three months; samples of reaction liquor were taken for analysis five times per week and feed wastewater analyzed at least twice per week.

RESULTS AND DISCUSSION

Batch Reactor Tests

The objectives of the batch reactor tests were to establish the following:

- Reaction order/type for each wastewater constituent;
- Reaction sequence; and
- Tolerance limits of constituents on individual reactions.

Reaction Order/Type Figure 2 shows typical biodegradation profiles for a) ammonia, b) cyanide, c) phenol and d) thiocyanate by sludge acclimated to synthetic wastewaters. These profiles indicate that ammonia, phenol and thiocyanate degradation reactions were zero order reactions, and they could be mathematically described as:

$$\frac{dC}{dt} = -k$$

where C = constituent concentration, mg/L
k = reaction rate, mg/L/hr.
t = time, hours

The cyanide degradation profile indicate the reaction is first order and it can be mathematically described by:

$$\frac{dC}{dt} = -kC$$

In activated sludge treatment facilities there is always a concern/question about the fate of cyanide (i.e., is removal due to biodegradation or air stripping). In experiments conducted in closed systems where air from the reactor was scrubbed with a sodium hydroxide solution to capture any hydrogen cyanide stripped, analysis and material balances showed that better than 99.9 percent of the cyanide was biodegraded. This analysis also revealed that the degradation of cyanide was stoichiometric (i.e., one mole of cyanide produced one mole of ammonia) according to the following expression:

$$CN^- + 0.5\,O_2 \xrightarrow{enzyme} CNO^- \xrightarrow{2H_2O} OH^- + NH_3 + CO_2$$

Material balance on the thiocyanate degradation reaction revealed that ammonia production also was stoichiometric (i.e., one mole of thiocyanate degraded to form one mole of ammonia).

Both batch and continuous flow reactor tests revealed that only free[a] cyanide is amenable to biodegradation; the complex cyanide species were unaltered. This observation is consistent with the earlier findings of Pettet and Mills (1954) who showed that cyanide degradability decreased as the stability of metallo cyanide complexes increased.

Reaction Sequence and Tolerance Figure 3 presents a typical illustration of the sequence in which the various biological reactions occur in batch treatment of coke plant wastewater. This figure shows biodegradation of both cyanide and phenol occurred simultaneously and proceeded without any lag, a characteristic of a well acclimated/developed sludge. These data also indicate that the microbial population tolerated the concentrations of constituents tested without any apparent inhibition (100 mg/L phenol exerted no inhibition on cyanide degradation and 20 mg/L cyanide exerted no inhibition on phenol degradation). However, both thiocyanate and ammonia oxidation proceeded only after significant lag periods which corresponded to the condition where the cyanide concentrations were reduced to about 3.0 mg/L for thiocyanate and 0.5 mg/L for ammonia (not shown in Figure 3).

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[a] As measured by ASTM Method D2036-82: Standard Test Methods for Cyanide in Water-Method C Weak Acid Dissociable Cyanides

The data in Figure 3 were generated for synthetic wastewater; however, similar reaction trends were observed in tests with real coke plant wastewater. In effect, the overall reaction sequence in a batch treatment situation would entail both phenol and cyanide being oxidized first, followed by thiocyanate and then ammonia oxidation.

This information suggests that in the operation of a continuous flow treatment that it will be imperative that low concentrations of cyanide be maintained in the aeration basin in order to achieve effective overall treatment of all parameters of concern, especially ammonia and thiocyanate.

Continuous Flow Reactors

The objectives of continuous flow reactor experiments were to determine the relationships between treatment effects and process operating conditions especially hydraulic residence time and sludge (microbial) concentrations (as measured by total volatile solids).

Tables 3 and 4 present the results of experiments conducted with synthetic and actual wastewaters. In all test runs, cyanide was degraded to concentrations less than 0.1 mg/L.

Assuming the cyanide degradation reaction in the continuous flow reactors progressed according to a first order reaction, then cyanide degradation in each reactor can be described by:

$$k = \frac{1}{HRT}\left[\left(\frac{Co}{C}\right) - 1\right]$$

where: Co = Feed cyanide concentration, mg/L
C = Effluent cyanide concentration, mg/L
HRT = Reactor hydraulic residence time, days
k = Cyanide degradation rate, $(\text{day})^{-1}$

and:

$$K = k(MLVS)^{-1}$$

where: K = Specific reaction rat, (mg/L•day)-1
MLVS = Reactor mixed liquor volatile solids concentrate, mg/L

Table 5 presents the reaction rates determined for each of the test runs shown in Tables 3 and 4. For each test run within a series (i.e., Series A,B and C), K was fairly constant. Average values for K are:

- Series B Synthetic wastewater without thiocyanate
  $K = 0.245\ (\text{mg/L}\cdot\text{day})^{-1}$
- Series A Synthetic wastewater with thiocyanate
  $K = 0.090\ (\text{mg/L}\cdot\text{day})^{-1}$
- Series C Real coke plant wastewater
  $K = 0.049\ (\text{mg/L}\cdot\text{day})^{-1}$

These data suggest that other constituents in the wastewater or metabolic products from their degradation could have exerted inhibitory effects on the cyanide degradation reaction. In the case of test Series A, thiocyanate and/or sulfide or sulfate (products of thiocyanate degradation) could be the inhibitory agents. In Series C (real wastewater), again thiocyanate and possibly other unmeasured constituents in the wastewater could be inhibitory agents.

CONCLUSIONS

Biological degradation of free cyanide in a complex industrial wastewater was examined in both batch and continuous flow reactors. This evaluation determined the following:

- Microbial degradation of cyanide progresses according to a first order reaction with respect to cyanide.
- Cyanide degrades to produce stoichiometric quantities of ammonia.
- Only free cyanide (as measured by ASTM) is biodegradable.
- Free cyanide inhibits the biodegradation of other constituents in the wastewater — ammonia oxidation (nitrification) was totally inhibited at cyanide concentrations greater than 0.5 mg/L and thiocyanate at concentrations greater than 3.0 mg/L.
- Other wastewater constituents and/or their metabolic products could exert competitive inhibitory effects on cyanide degradation.
- Microbial degradation of thiocyanate progresses according to a zero order reaction and it produces stoichiometric quantities of ammonia.

ACKNOWLEDGEMENT

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TABLE 1

Composition of coke plant wastewaters and effluent discharge limits treatment must attain

Parameter	Concentration (mg/L)	
	Wastewater(a)	BATEA Limits(b)
Ammonia	1800 - 6500	25 (95)
Cyanide	10 - 200	5.5 (10)
Oil and Grease		17 (51)
Phenols (4AAP)	400 - 3000	0.05 (0.10)
Sulfide	200 - 600	
Suspended Solids		205 (397)
Thiocyanate	100 - 2500	
Total Dissolved Solids	4000 - 13000	
Benzene	5.4	(0.05)
Napthalene	0.8	(0.05)
Benzo-a-pyrene	0.1	(0.05)
pH	7.5 - 9.1	

(a) Compiled from reports by Biczysko and Suschka (1966); Kostenbader and Flecksteiner (1969); Jablin and Chanko (1972); Barker and Thompson (1973); Wong-Chong *et al.*(1978); and Wong-Chong *et al.*(1985).

(b) Best Available Technology Economically Achievable, concentration based on 155 gallons of wastewater per ton of coke produced, 30-day average and one day maximum ( ).

TABLE 2

Composition of synthetic coke plant wastewaters

Wastewater volume	20.0 L
Dibasic ammonium phosphate	20.0 gms
Potassium cyanide	10.0 gms
Potassium thiocyanate	20.0 gms
Phenol	23.5 gms
Calcium Chloride	0.6 gms
Ferric Chloride	0.2 gms
Magnesium Sulfate	0.6 gms
Sodium Chloride	27.0 gms
pH adjusted to	9.5 to 10.5

TABLE 3

Operation and performance of continuous flow reactor treating synthetic coke plant wastewaters

Test Run	Operating Conditions			Treated Water Quality (mg/L)			
	HRT[a] (days)	MLVS[b] (mg/L)	pH	Ammonia	Cyanide	Phenol	Thiocyanate
Feed			9.5-10.5	250	182	1,121	522
A1	5.9	2,600	6.8	239	0.1	<1	5.7
A2	4.1	5,700	6.1	179	<0.1	<1	<1
A3	2.9	7,300	7.1	144	<0.1	<1	<1
A4	2.3	10,200	6.5	71	<0.1	<1	<1
Feed			9.0	204	165	1,103	0
B1	2.0	3,000	6.6	96	<0.1	<1	
B2	1.3	5,700	6.9	110	0.1	<1	
B3	1.0	6,900	6.8	88	<0.1	<1	

[a] Hydraulic retention time = Reactor volume Wastewater feed rate

[b] Reactor mixed liquor volatile solids concentration

TABLE 4

Operation and performance of continuous flow reactor treating coke plant wastewaters

Test Run	Operating Conditions			Treated Water Quality (mg/L)			
	HRT[a] (days)	MLVS[b] (mg/L)	pH	Ammonia	Cyanide	Phenol	Thiocyanate
Feed			9.7	93	82	1,113	524
C1	1.8	8,630	7.1	25	<0.1	<1	<1
C2	1.3	9,700	7.1	61	<0.1	<1	<1
Feed			9.7	249	72	1,163	607
C3	2.3	12,800	7.1	70	<0.1	<1	<1

[a] Hydraulic retention time = Reactor volume Wastewater feed rate

[b] Reactor mixed liquor volatile solids concentration

TABLE 5

Microbial cyanide degradation rate

Test Run	HRT (days)	MLVS (mg/L)	$k^{(a)}$ (day)$^{-1}$	K$^{(b)}$ (mg/L•day)^{1}	Comments
A1	5.9	2,600	308.3	0.119	Synthetic wastewater containing ammonia, cyanide, phenol and thiocyanate Average K = 0.090 (mg/L•day)$^{-1}$
A2	4.1	5,700	443.7	0.078	
A3	2.9	7,300	627.2	0.086	
A4	2.3	10,200	790.9	0.078	
B1	2.0	3,000	824.5	0.275	Synthetic wastewater without thiocyanate Average K = 0.245 (mg/L•day)$^{-1}$
B2	1.3	5,700	1,268.5	0.222	
B3	1.0	6,900	1,649	0.239	
C1	1.8	8,630	455.0	0.053	Actual wastewater Average K = 0.245 (mg/L•day)$^{-1}$
C2	1.3	9.700	630.0	0.065	
C3	2.3	12.800	356.1	0.028	

(a) $k = 1/HRT\ [Co/C - 1]$

(b) $K = k(MLVS)^{-1}$

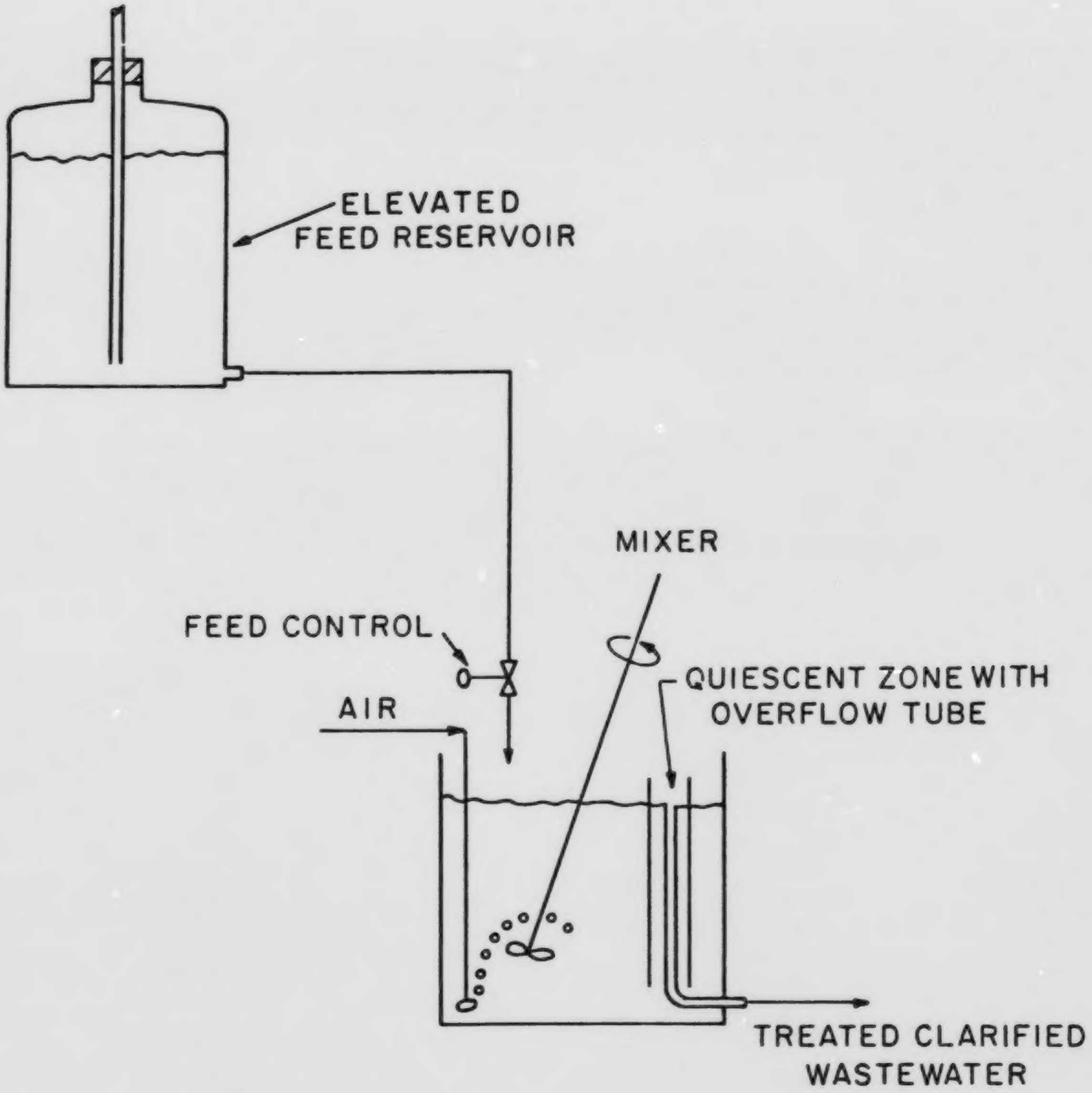
ELEVATED
FEED RESERVOIR
MIXER
FEED CONTROL
QUIESCENT ZONE WITH
OVERFLOW TUBE
AIR
TREATED CLARIFIED
WASTEWATER

Fig. 1 Schematic flowdiagram of continuous flow reactor

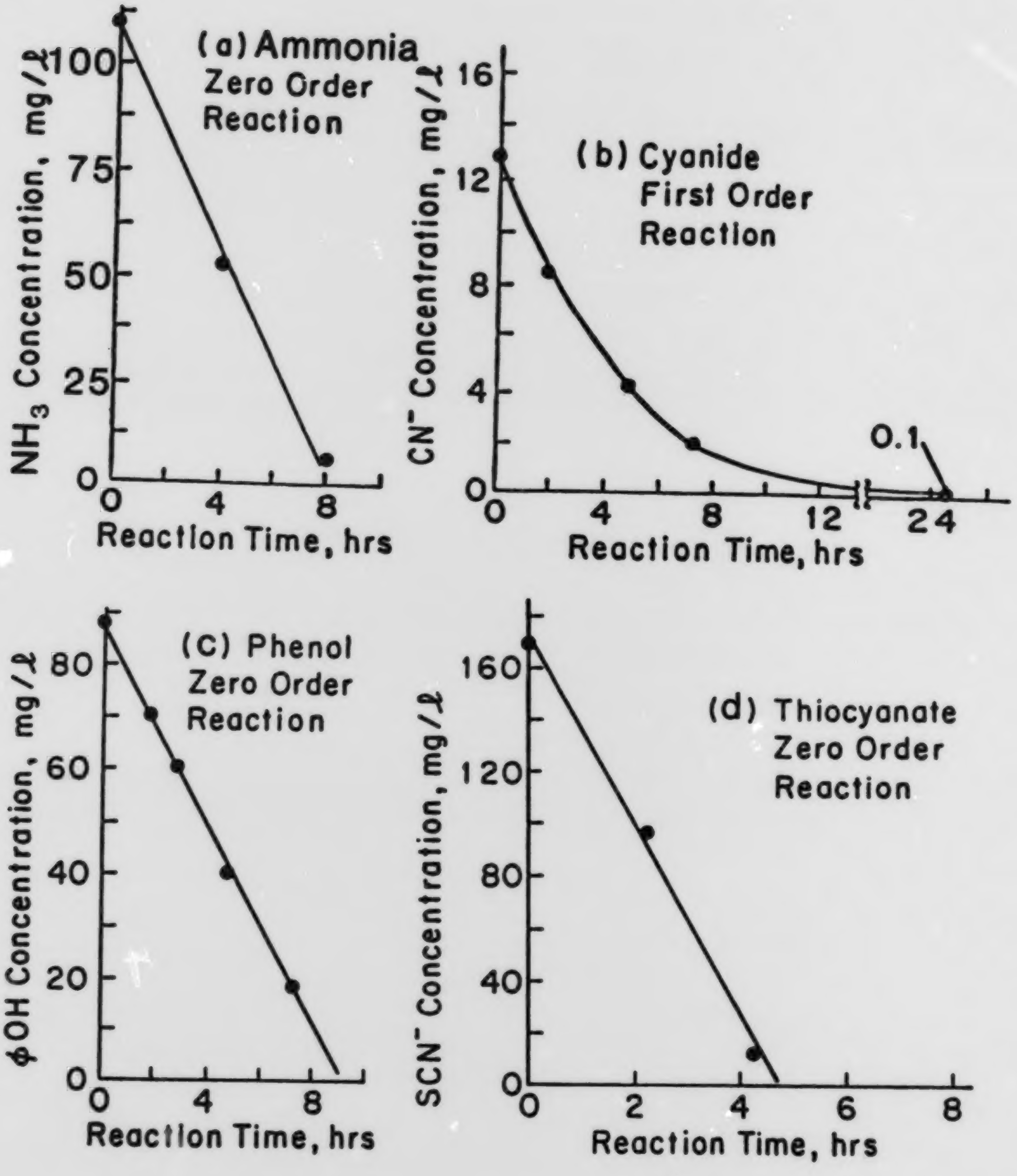

Fig. 2 Biodegradation profiles of individual constituents in coke plant wastewater

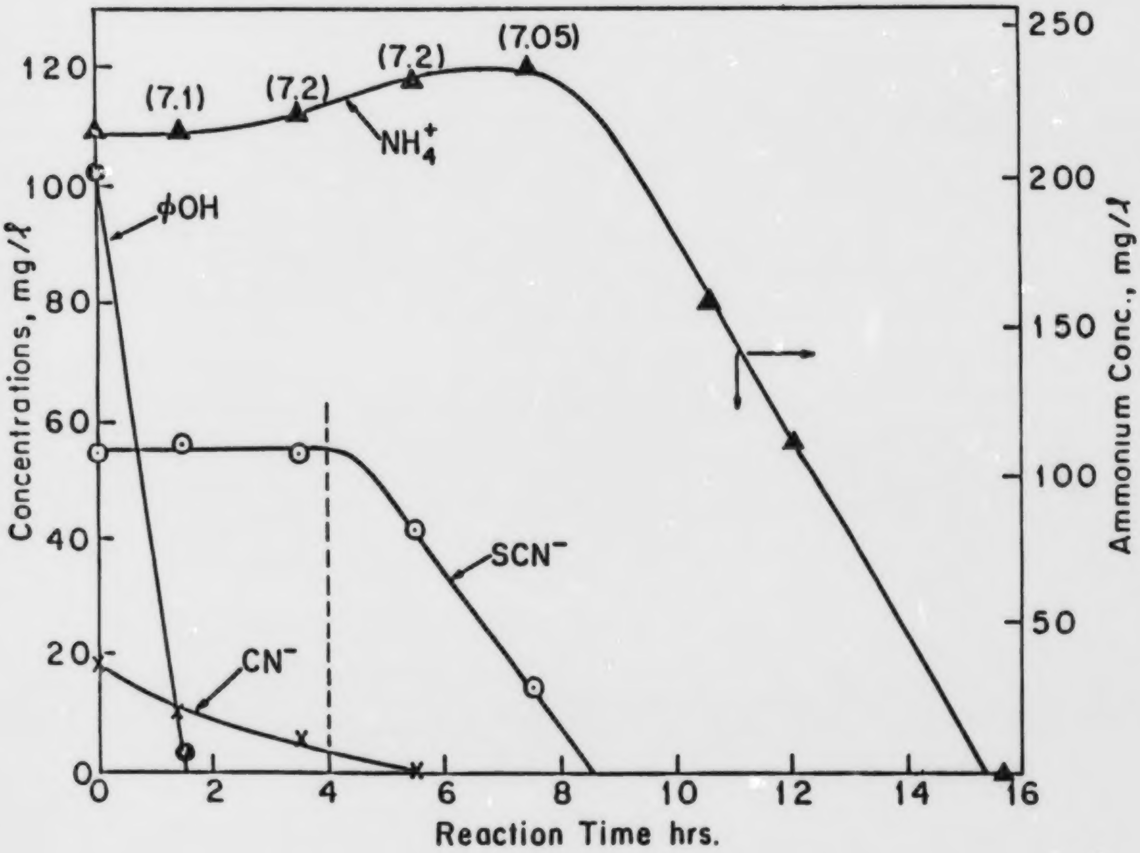

Fig. 3 Typical degradation sequence for ammonia, cyanide, phenol and thiocyanate in a batch reactor

REACTIONS OF PRECIOUS METAL COMPLEXES WITH BIOPOLYMERS

by

J.J. BYERLEY, J.M. SCHARERI AND S. RIOUX
Department of Chemical Engineer Waterloo University, Waterloo, Ont. Canada

ABSTRACT

The sorption of silver and gold complexes by biopolymers (alginate, chitosan), four algal species (*C. vulgaris, C. pyrenoidosa, C. kessleri* and *S. obliquus* biomass was investigated. Sorption characteristics (efficiency and affinity) depended on the nature of the metal complex as well as on the type of sorbent used. Silver ion (Ag^+) sorbed most strongly by chitosan (194 mg Ag^+/g sorbent as observed maximum), while sorption on alginate and algal biomass was poor. At equilibrium concentration of 10 mg/L Ag^+, the sorption capacity was 90 mg Ag^+/g (dry weight) with chitosan, 20-30 mg Ag^+ (dry weight) with the algae, and less than 10 mg Ag^+/g with alginate as the sorbing agent. In general the cyano complex of silver, $Ag(CN)_{-2}$ was not adsorbed as well, maximum values were observed with chitosan (45.6 mg/g). Among the sorbents used, chitosan was shown to be the best sorbent for gold(III) chloride ($AuCl_4$) complexes, followed closely by algae. Alginate showed little affinity for $AuCl_4$. The sorption of $AuCl_4$ by chitosan was strongly pH dependent, maximum sorption (99.5%) occurred at acidic pH values (pH 3.0). Gold cyanide, $Au(CN)_{-2}$ sorption from alkaline solutions was significantly less, approximately 30 mg Au/g chitosan was observed. Sorption studies with *C. kessleri, C. pyrenoidosa,* and *S. obliquus* did not show promise for adsorbing gold cyano complexes. Department of Chemical Engineering, University of Waterloo, Waterloo, Ontario.

RÉACTION DE COMPLEXES DE MÉTAUX PRÉCIEUX AVEC DES BIOPOLYMÈRES

J.J. Byerley, J.M. Scharer et S. Rioux

Département de Génie Chimique
Université de Waterloo
Waterloo (Ontario) Canada

RÉSUMÉ

La sorption des complexes aurifères ou argentifères par des biopolymères (alginate, chitosane) et par quatre espèces d'algues (*C. vulgaris, C. pyrenoidosa, C. kessleri* et *S. obliquus*) a été étudiée. Les caractéristiques de sorption (efficacité et affinité) dépendent à la fois de la nature du complexe métallique et du type de sorbant utilisé. La sorption de l'ion argent (Ag^+) par le chitosane est très efficace (valeur maximale observée : 194 mg Ag^+/g sorbant) alors que celle effectuée par l'alginate et par les algues est assez faible. À une concentration d'équilibre de 10 mg/L Ag^+, la capacité de sorption est de 90 mg Ag^+/g (poids sec) pour le chitosane, de 30 mg Ag^+/g pour les algues et moins de 10 mg Ag^+/g pour l'alginate. En général, la sorption de l'argentocyanure $Ag(Cn)_2$ est plus faible, la sorption maximale observée par le chitosane est de 45,5 mg/g. De tous les sorbants utilisés, le chitosane est celui qui présente les meilleures capacités de sorption des aurochlorures ($AuCl_4$), suivi de près par les algues. Les alginates présentent peu d'affinités pour $AuCl_4$. La sorption de $AuCl_4$ par la chitosane dépend en grande partie du pH, la sorption maximale (99,5 %) se produisant à des pH acides (pH 3.0). La sorption de l'aurocyanure $Au(Cn)_2$ dans des solutions alcalines est plus faible, soit 30 mg Au/g de chitosane. Les études de sorption montrent que la sorption des aurocyanures par *C. kessleri, C. pyrenoidosa* et *S. obliquus* est très faible.

INTRODUCTION

Microorganisms are known to sequester metal ions from solutions. High metal sorption capacities have been reported for various bacteria (Beveridge 1986; Norberg and Persson, 1984; Friis and Myers-Keith, 1986), fungi and yeasts (Strandberg *et al.*, 1981; Tzesos, 1985; Tobin *et al.*, 1984), and for salt water and fresh water algae (Sakaguchi *et al., 1978; Horikoshi et al.*, 1979; Nakajima *et al.*, 1981). In some cases, the reported metal uptake was equal to or exceeded the capacity of conventional ion exchange resins used in various hydrometallurgical processes. Most of these studies, however, addressed the sorption of transition metal ions or the uranyl ion from synthetic solutions. The sorption capacity from process solutions was significantly less (Byerley *et al.*, 1987). In addition to the lower capacity, the lack of metal specificity and poor biomass reuse potential preclude the large-scale, industrial use of biomass for transition metal or uranium recovery.

Studies concerning the biosorption of precious metal ions by algae have been more promising (Hasea *et al.*, 1986; Darnall *et al.*, 1986). The authors reported essentially complete removal of gold and silver from synthetic solutions. In addition to removal from the aqueous phase, a significant fraction of the gold(III) ion was apparently reduced to elemental gold on the cell surface. This reduction obviates the need for cementation with zinc or electrolyte reduction. Gold and silver sorption was reported to be essentially independent of the pH (Darnall *et al.*, 1986). This is in contrast with the biosorption of transition metal ions and uranium, which are known to be strongly pH dependent. Algae could be grown inexpensively and harvested from tailings ponds during the summer season. Thus, algae could provide an attractive means of precious metal recovery from the economic and the environmental perspective.

Most previous studies of gold and silver biosorption were empirical in nature; at the present neither the primary sorption mechanism nor the kinetics of the sorption process is well understood. In hydrometallurgy, gold(I), gold(III), and silver(I) ions are classified as soft ions for their ease of gaining electrons. It is this property that allows them to form inner sphere complexes with soft donor ligands such as sulphides, thiourea, amino groups, and cyanides. The formation of the exceptionally stable cyano complexes is exploited by the gold and silver mining industry of recovery of metal values (Osseo-Asare *et al.*, 1989). Sulphide, amine, and carboxyl amine residues are common features of biopolymers, hence, complexation on biosurfaces is not unlikely. We report the results of a comparative study of silver and gold biosorption by various algae and biopolymers. The main purpose of the study was to examine the kinetic and mechanistic aspects of biosorption with the view of secondary metal recovery from barren solutions, small scale in situ leachates, and waste streams from electroplating and other metal finishing operations.

MATERIALS AND METHODS

Sorption studies were performed with unicellular algae *Chlorella vulgaris* ATCC 30581, *Chlorella kessleri* UTEX 263, *Chlorella pyrenoidosa* UTEX 26, and *Senedesmus obliquus* UTEX 78). Gold and silver sorption by bacteria have been reported previously (Byerley *et al.*, 1989). The species designated by UTEX were obtained from the University of Texas algal repository. The algae were propagated heterotrophically in modified Bristol medium at 23°C, pH 7.0 on 1.5% agar surfaces as described previously (Byerley *et al.*, 1989). The confluent algal mass was harvested by scraping with a sterile spatula and stored at 4°C for no longer than 14 days. The moisture content was determined by drying approximately 100 mg fresh weight algal mass for 12 hours at 103°C. Viable algae were used in most experiments. The results, however, were reported on a dry weight basis. Dry weights were calculated by correcting for the moisture content.

Parallel sorption studies were performed with alginate and chitosan. These biopolymers were selected for their ease of availability and low cost. Alginate is a negatively charged polysaccharide (6-carboxy-glucan) having a pK value of 4.2. The negative charge is due to the carboxylic residues on the glucosidic backbone. It occurs as a capsular slime in many algal species and in a few bacteria. In contrast, chitosan (2-amino-deoxy-glucan) carries a positive charge due to the free amino residues. Its physico-chemical and chelating properties have been described by Muzzarelli (1985). The characteristic pK value of chitosan depends on the density of the amino groups and is usually between 6.2 and 7.0. A fraction of the amino groups is always acetylated. The fully acetylated form is known as chitin. These aminoacetyl residues are said to be coordinating with some metal ions and complexes. It is interesting to note, that the natural abundance of chitosan- type material is said to be second only cellulose (Muzzarelli, 1985). The chitosan (lot No. 46F-0268) used in this study was 67% deacetylated derivative of crab chitin. Alginate and chitosan were obtained from Sigma Chemical Co.

Reagent grade gold(III) chloride, and silver(I) nitrate were obtained from J.T. Baker Co. Gold(I) cyanide solutions were prepared by dissolving elemental gold in sodium cyanide/hydrogen peroxide solutions at pH 11.0. The concentrations of other dissolved metal ions, including gold and silver, were determined by atomic adsorption spectrometry.

Both dynamic and equilibrium-type experiments were performed. The dynamic experiments involved the suspension of 100 mg dry weight of biomass (or biopolymer) in 100 mL test solution containing a known concentration of metal ions. The suspension was placed immediately on a Burrell wrist action shaker operated at 300 rpm, 7° angular displacement. The solution was sampled periodically with a microsyringe equipped with a 0.45 micrometer micropore filter. The case of equilibrium studies, the procedure was identical until the first sample. After the first sample, 100 mL fresh solution was added and the test solution was re-sampled after a prescribed time period. The procedure was repeated until a total volume of a 300 mL fresh solution was added. Equilibrium conditions were normally achieved after 2 hours of incubation with silver solutions. Gold solutions, however, required incubation periods of 6 hours or more. Usually, three different concentrations of metal solutions were employed for each metal species/adsorbent combination. The pH of the solutions was determined routinely in each experiment. Unless indicated otherwise, the tests were performed at ambient temperatures. All tests were carried out in duplicate with control flasks of known metal concentrations but without biomass.

RESULTS AND DISCUSSION

The precious metal sorbates included the cationic Ag^+ and the anionic $AgCN_{-2}$, $AuCl_{-4}$, and $AuCN_{-2}$ complexes. A number of dynamic experiments were performed. Typical results with chitosan as the sorbent are shown in Fig. 1. The kinetic profile of Ag^+ sorption was similar to the profiles reported previously for hard metal ions such as $UO^{2+}{}_2$ with alginate, bacteria, and fungi (Byerley *et al.*, 1987). It has been shown that if the sorption sites are non-cooperative, the kinetics of the sorption process can be described by the following differential equation:

$$\frac{dX}{dt} = k_+ \{(X_{max} - x)\, c - Kx\}$$

where x = sorbed metal ion (mg/g sorbent dry weight)
x_{max} = maximum sorption capacity (mg/g sorbent)
C = metal ion concentration in the liquid
k_+ = kinetic rate constant in the forward (sorption direction)

For process application, it is clear that the desirable properties of the sorbent include a fast forward kinetic rate constant coupled with a high maximum sorption capacity and a low dissociative equilibrium constant. The estimated kinetic rate constant ranged from 7×10^{-2} to 11×10^{-2} $mM^{-}1\ h^{-1}$ (8 to 10 $(mg/L)^{-1}h^{-1}$), which compared favourably with sorption rate constants for the uranyl ion using bacteria and fungi as sorbing substances.

During our attempts to model gold sorption it became obvious that the more complex gold uptake profiles could not be represented by a sorption-type kinetic model. Gold(III) and gold(I) ions have been reported to undergo reduction to elemental gold during biosorption (Darnall *et al.*, 1986). In our hands, the whitish, oatmeal-like flakes of chitosan became yellow initially and later purple in the presence of gold solutions. Algae also assumed similar purple colouration. This characteristic colour change has been noted previously to be reminiscent in the "purple of Cassius" (Darnall *et al.*, 1986) that is often observed with colloidal gold. In addition, gold sorption was, to a large extent, irreversible and could not be recovered without the destruction of the biomass.

Mindful of the reductive processes in the case of gold solutions, a second differential equation was introduced for expressing this phenomenon, as a first order process, as follows:

$$\frac{dX_{RED}}{dt} = k^* \frac{C}{\sigma} \tag{2}$$

where X_{RED} = reduced gold on the sorbent surface (mg/g)
k^* = reduction rate constant
σ = sorbent concentration (g/L)

The uptake of gold by either sorption or chemical reduction is constrained by the following material balance:

$$\sigma(X_{RED} + X) + C = C_o \tag{3}$$

where C_o is the initial concentration of the gold ion in solution

The theoretical curves generated by numerical integration of either equation (1) (sorption only) of the simultaneous integration of equations (1) and (2) (sorption and reduction) are shown in Fig. 2. The integration was carried out by a computer algorithm based on orthogonal collocation. The resemblance between the theoretical curves and the experimental results given in Fig. 1 is evident. An important assumption concerning the combined (adsorption and reduction) model is that sorption and reduction occur at separate sites on the biomass. Thus, the two processes occur independently, i.e., the sorption sites are not blocked by the reduction of gold. Although this model is tentative, it seems to agree with the experimental results.

The theoretical isotherms generated by plotting the metal sorption on the solid against the concentration in the liquid phase are shown in Fig. 3. Strictly speaking, the sorption curves derived for reduction processes are not true isotherms, since equilibrium is never reached. However, near equilibrium states are expected to be achieved after 24 hours of simulated exposure. The curves were generated for illustrative purposes only and the magnitudes do not reflect experimentally observed differences. The profiles for sorption only and for sorption coupled with reduction processes are surprisingly similar. An interesting feature of these curves is that the Langmuir-type isotherm of pure sorption phenomenon becomes a Freundlich-type isotherm for sorption/reduction. Each type of isotherm requires the estimation of two parameters from the experimental observations. However, the experimental observations were usually far too scattered for performing rigorous model discrimination on the basis of the parameter estimates. If chemical reduction is the predominant means of metal uptake, the "isotherm" becomes essentially a straight line with very little curvature.

The biosorption equilibria of silver(I) from silver nitrate solutions with chitosan are shown at test pH values of pH = 3.5 and pH = 6.5 in Fig. 4. At these pH values silver occurs at monovalent cation (Ag^+) in nitrate solutions. The relatively high silver sorption capacity of chitosan (calculated maximum sorption capacities of 129 mg/g at pH = 6.5 and 198 mg/g at pH = 3.5) is remarkable. This probably reflects the complexation of the silver ion with amino residues of chitosan. Amino nitrogen is a well known donor ion in complexation reactions with silver. The apparent pH dependence of the sorption reaction is in variance with the observations of Darnall *et al.* (1986). It would appear that the pH effect reflects the ease of electron donation by the amino ligand which, of course, is favoured by the lower pH. In contrast, the carboxylic groups in alginate do not seem to form stable complexes with monovalent silver even though ionic interactions between the positively charged Ag^+ and the negative carboxylic residue would be advantageous. Maximum silver sorption on alginate was 14.7 mg/g at pH

6.0. The lack of effective binding of the soft transition metal ions with carboxylic residues is well known and may explain the results in the present case. The sorption of Ag^+ by the algae was intermediate between chitosan and alginate. The alga, *C. pyrenoidosa* adsorbed a maximum of 50.8 mg/g, while *C. kessleri* and *S. obliquus* sorbed 45 mg/g at pH 5.8 to 6.0 from solutions containing up to 50 mg/L Ag^+.

The sorption of silver(I) from cyanide solutions was significantly less than from nitrate. These experiments were carried out at alkaline pH values ranging from pH 9.7 to 10.4. The predominant species of silver in these solutions was the cyanide anion $Ag(CN)^-_2$. As expected, the silver cyanide complex sorbed better in suspensions of chitosan than in the negatively charged alginate gel. Maximum sorption with chitosan was 45.6 mg/g sorbent in comparison to 17.8 mg/g with alginate. Of the algae, best results were obtained with *C. vulgaris*, which sorbed 28.8 mg/g of biomass.

The experimental results of gold(III) uptake as gold chloride ($AuCl^-_4$) complex is shown in Fig. 5. Of the sorbents tested, gold uptake by chitosan was particularly significant. Undoubtedly the high metal uptake was the result of the combination of Langmuir-type sorption and metal reduction. From solutions ranging in pH from 3.3 to 4.2 the maximum observed gold sorption was in excess of 150 mg/g chitosan. In comparison, *C. vulgaris* sorbed approximately 120 mg/g gold.

The pH is often regarded as the master variable in biosorption. This is largely due to the weakly acidic or weakly basic nature of the biopolymers. In fact, they may be regarded as weak acid/base ion exchange resins. The pH dependence of the uptake of the gold(III) chloride complex on chitosan is shown in Fig. 6. The metal uptake capacity of chitosan was essentially constant up to a pH of 5.0. Above this pH, the sorption capacity rapidly declined. It is interesting to note, that the sorption pattern essentially parallels the titration curve for chitosan. The pK value of chitosan used in this study was near 6.5. At this pH, 50% of the amino groups remained protonated. This corresponds to an approximate 50% reduction in sorption capacity. At pH = 10, virtually no protonated groups remained; the sorption capacity for negatively charged gold chloride complexes declined to essentially zero accordingly.

The sorption isotherm of $AuCl^-_4$ complexes with various algal species is illustrated in Fig. 7. Unlike silver, the uptake of gold by algae was slow and the experiments often continued for several days. The "equilibrium" data were quite scattered, which was a characteristic feature of experiments with viable algae. The lack of well defined sorption profiles reflect, in our opinion, the predominantly reductive chemical processes occurring on the algal surface. This was evidenced by the gradual colour change from green to purple with contact exposure time. The near linear isotherms were characteristic of sorption data when algae were used as the sorbing biomass and the profiles were consistent with the proposed model.

The uptake of gold(I) from cyanide solutions $AuCN^-_2$ was substantially lower than from any other complex tested. At pH 10.0, the uptake of gold(I) was approximately 8 mg/g with chitosan and 6 to 12 mg/g with algae as sorbents. Somewhat higher metal uptake (14 mg/g) was observed at pH 8.0 to pH 9.0. This reflects the exceptionally high stability of the gold cyanide complex which imparts resistance to uptake by ion exchange or complexation mechanisms (Osseo-Assare *et al.*, 1984). It seems that a polymer having more basic functional groups than the amino groups in chitosan is required for effective gold cyanide binding (Schwellnus and Greene, 1988). In addition, one may suppose that cyanide, being an effective inhibitor of electron transport processes in viable cells, blocks the reduction of gold, hence metal uptake by reductive mechanism.

An important aspect of the application of biopolymers is their affinity for the target metal in the presence of other metal ions and complexes. For example, fungal and some bacterial biomass was shown to be a good sorber of the uranyl ion (a hard ion). However, ferric ion (also a hard ion) competed successfully for sorption sites (Byerley *et al.*, 1987). In Fig. 8, the effect of the initial zinc to silver molar ratio on the molar mass ratio of the sorbed metals on chitosan is shown. The dotted line corresponds to one-to-one molar mass ratio. Since the equilibrium points are all above the line, this indicates that silver is preferentially sorbed on chitosan. For example, an 8:1 initial silver to zinc ratio resulted in 23:1 sorbed silver to zinc ratio. Similar results were obtained with copper (Cu^{2+}) as the competing cation. Although the preferential sorption pattern was not as pronounced as in the case of zinc, silver was taken up preferentially at any concentration ratio tested. There is some evidence that silver sorbes to sites that are not available to either copper or zinc. This is indicated by the lack of proportional reduction of silver when either copper or zinc is present in the solution. Although the equilibrium capacity for silver is reduced, the reduction is less than expected by simple replacement of silver ions by competing ions at the sorption sites. Experimental work concerning the selectivity of gold in solutions containing competing cations is currently underway.

CONCLUSIONS

The above studies comprise a preliminary identification and evaluation of the mechanisms and the kinetics of precious metal ions uptake by algal biomass and readily available biopolymers such as chitosan and alginate. The latter substances are often found as components of microbial surfaces. For the most part, the sorption of silver followed a Langmuir-type sorption model. The uptake of the gold complexes was more complicated. Experimental observations emphasized the importance of the reduction of gold ions on the biomass surface and its contribution to the sorption process. The biosorption results reflected the complexities of the process. Chitosan, for example, was a good sorber of silver ion, silver cyanide, and gold chloride complexes. The sorption of gold cyanide, however, was not remarkable. These seemingly curious results are probably due to the nature of the metal-ligand interactions and the affinity of the functional group on the sorbent. The sorption of silver on chitosan may involved both free amino and unprotonated aminoacetyl and hydroxyl groups. The latter may allow the dissociation of the silver cyanide and chelation with the uncharged groups. In contrast, the gold cyano complex is exceptionally stable. This stability coupled with low chelation potential results in poor sorption on any biosurface. Cyanide is known to inhibit biological electron transport, thus the biochemical reduction of gold cannot take place.

An important conclusion is the apparent difficulty of predicting biosorbent performance. It is clear, that substantially more research is needed for full understanding of the biosorption processes.

ACKNOWLEDGEMENT

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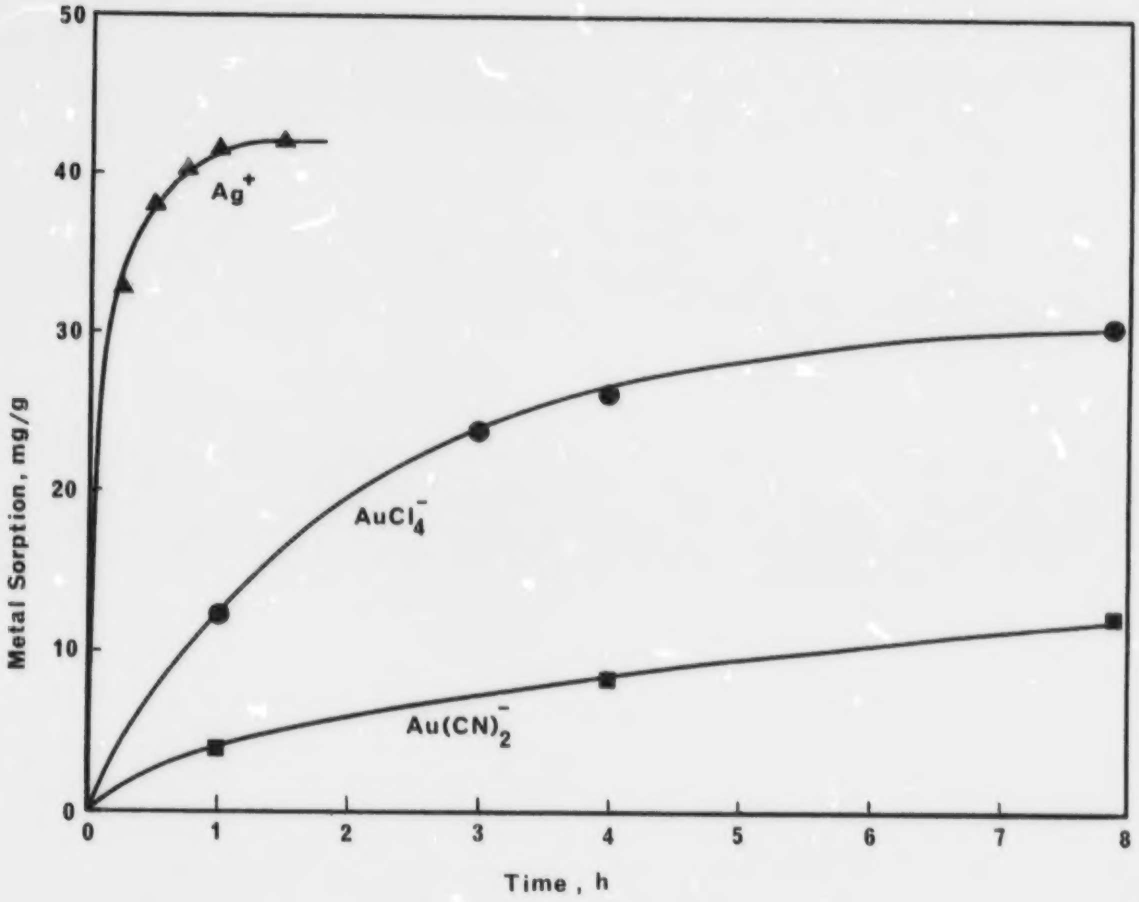

Fig. 1 Kinetics of silver(I) (Ag^+), gold(I) ($Au(CN)_2^-$) and gold(III) ($AuCl_4^-$) sorption by chitosan.

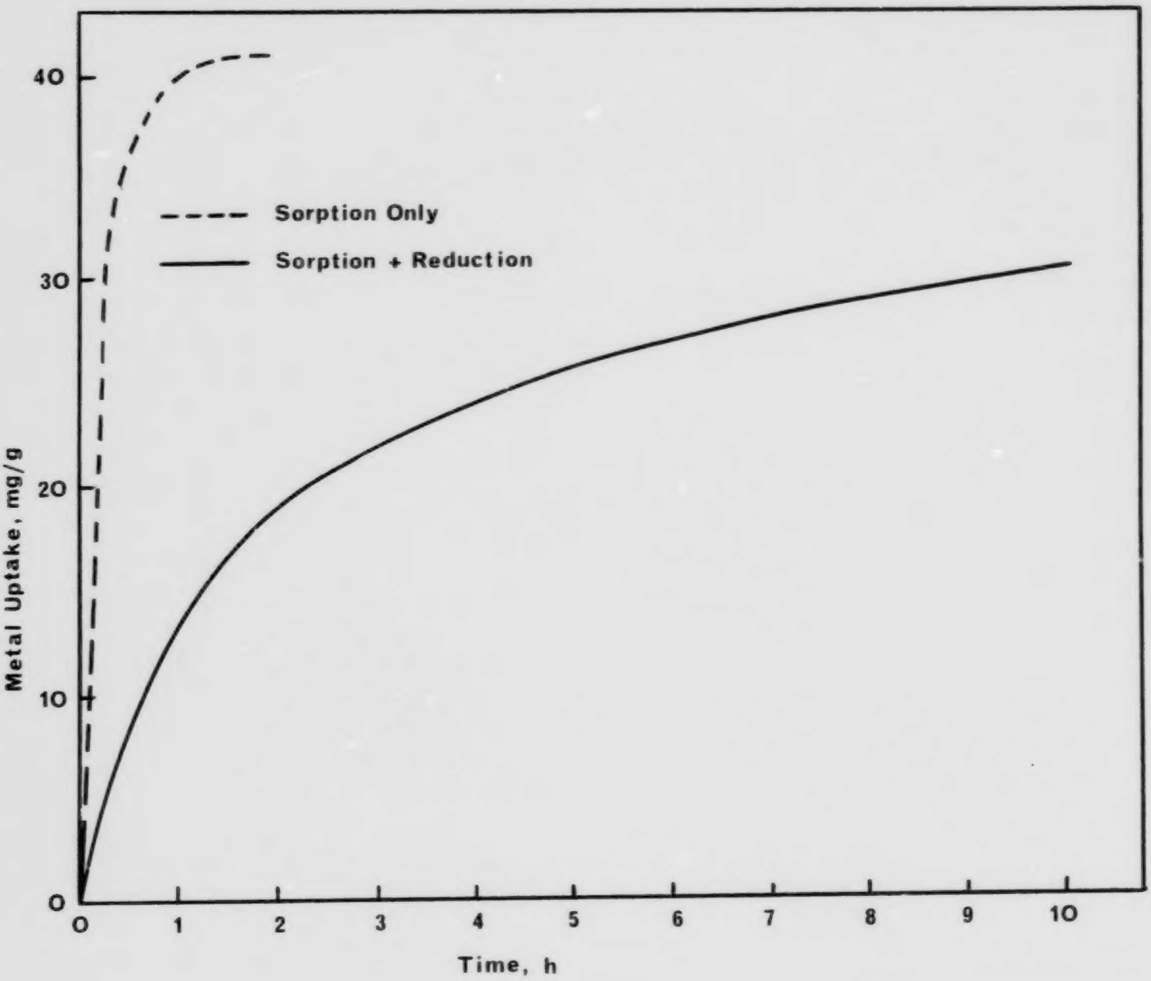

Fig. 2 Kinetic model of metal uptake by sorption and reduction.

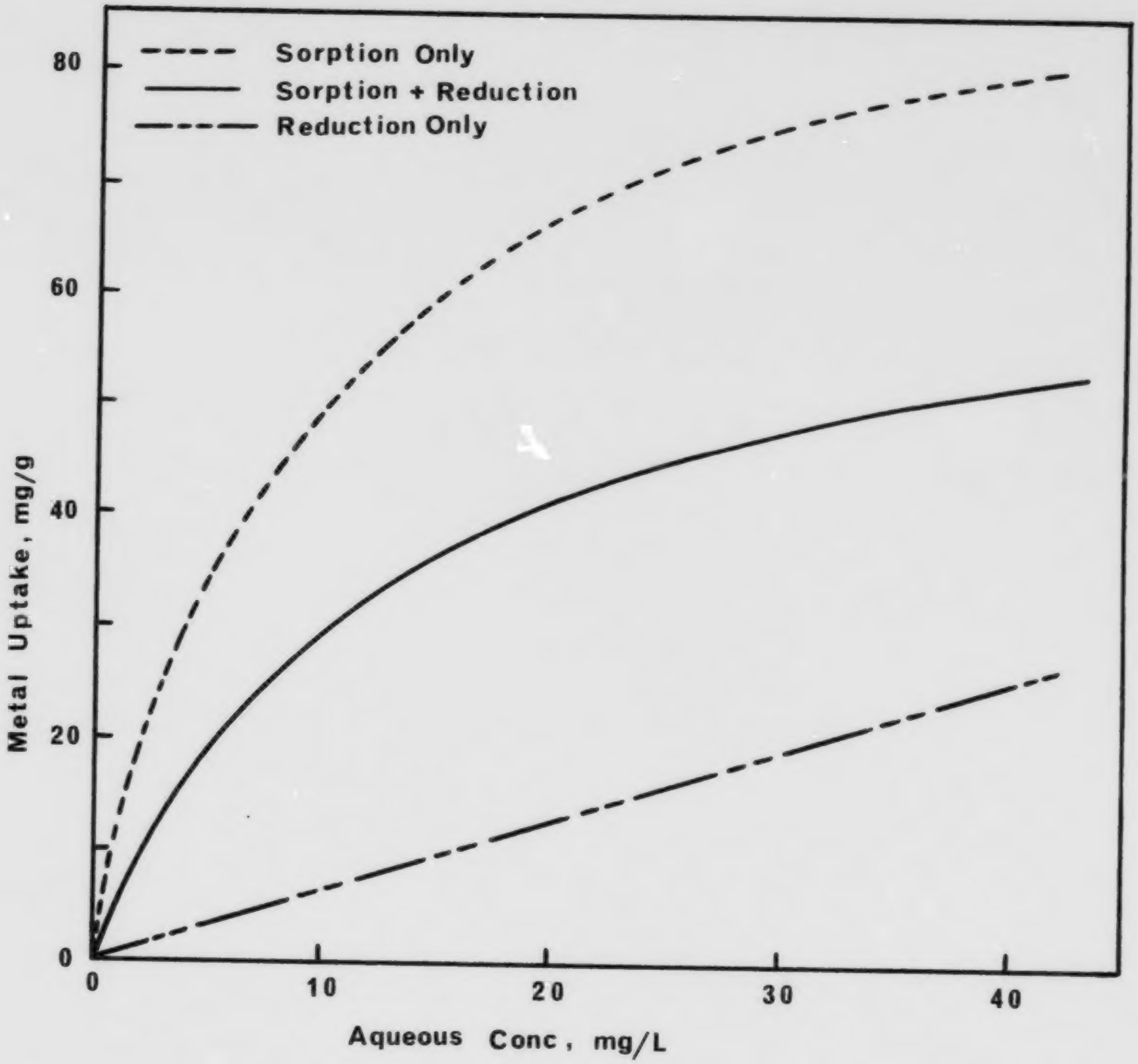

Fig. 3 Metal uptake models resulting from sorption and reduction mechanisms.

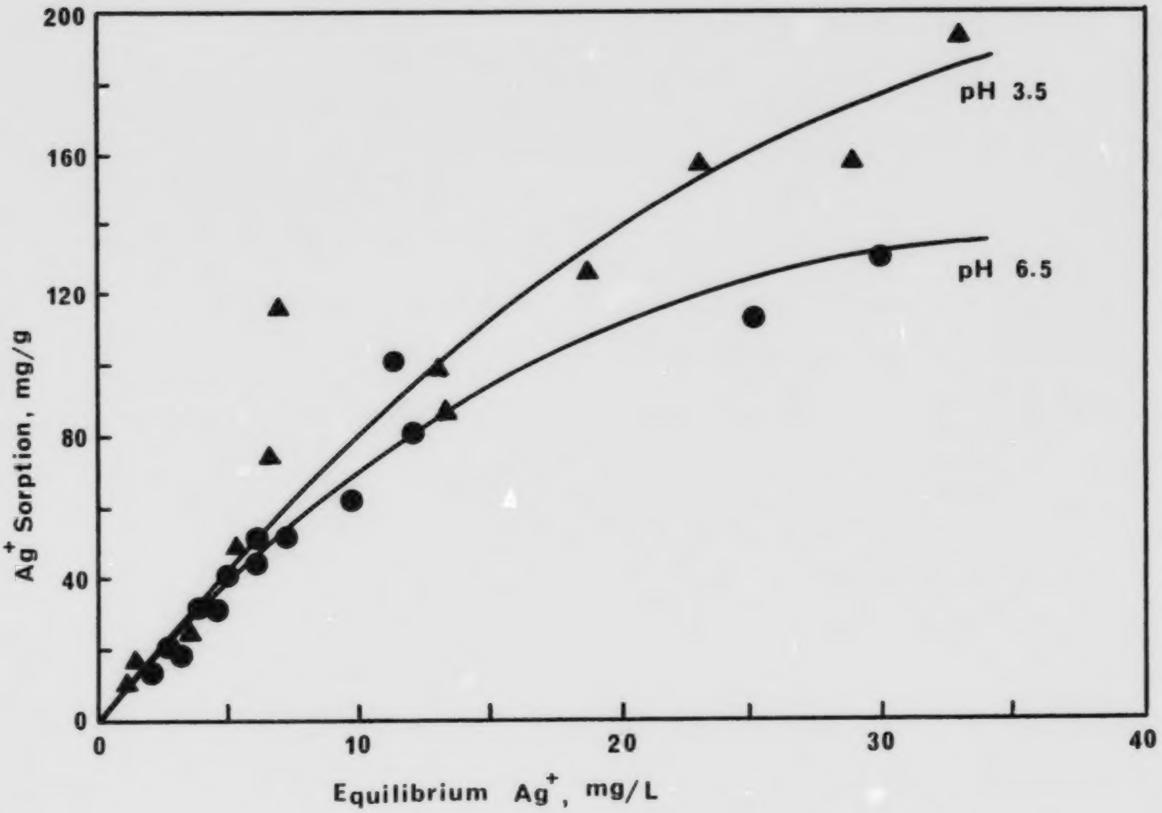

Fig. 4 Silver(I) (Ag^+) sorption isotherm with chitosan.

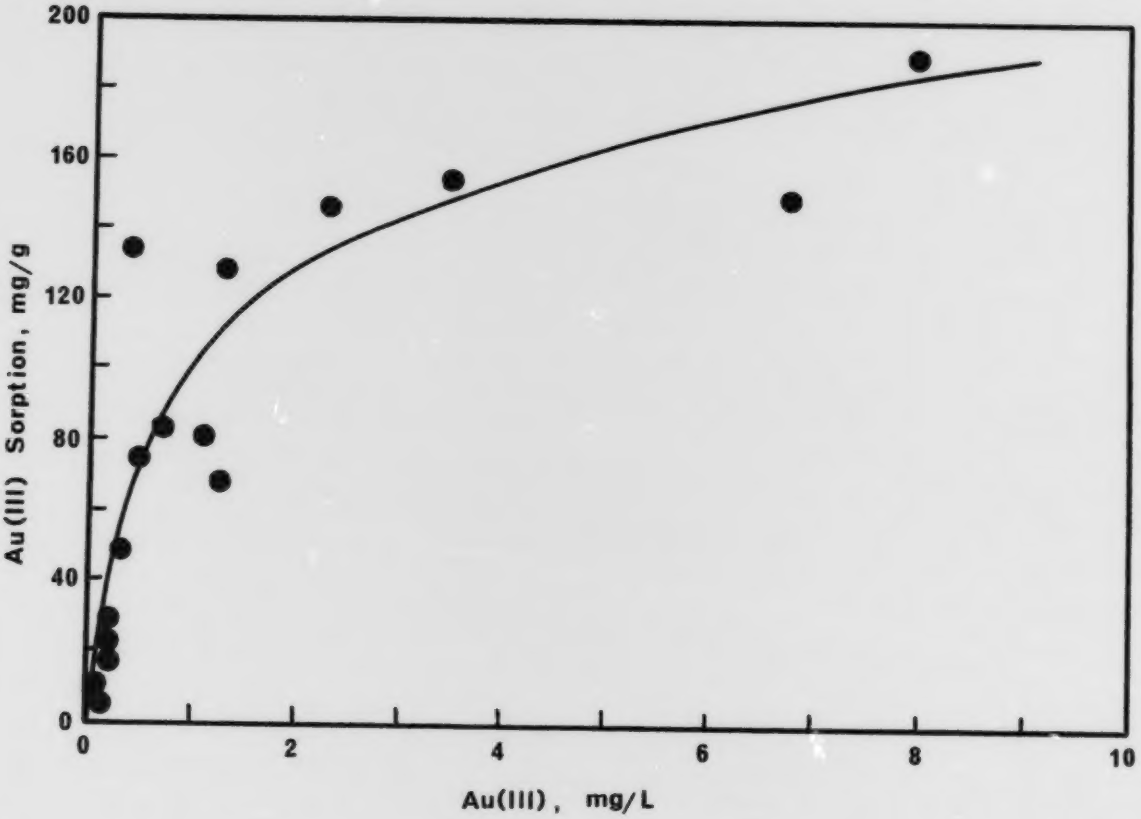

Fig. 5 Gold(III) ($AuCl_4^-$) sorption isotherm with chitosan.

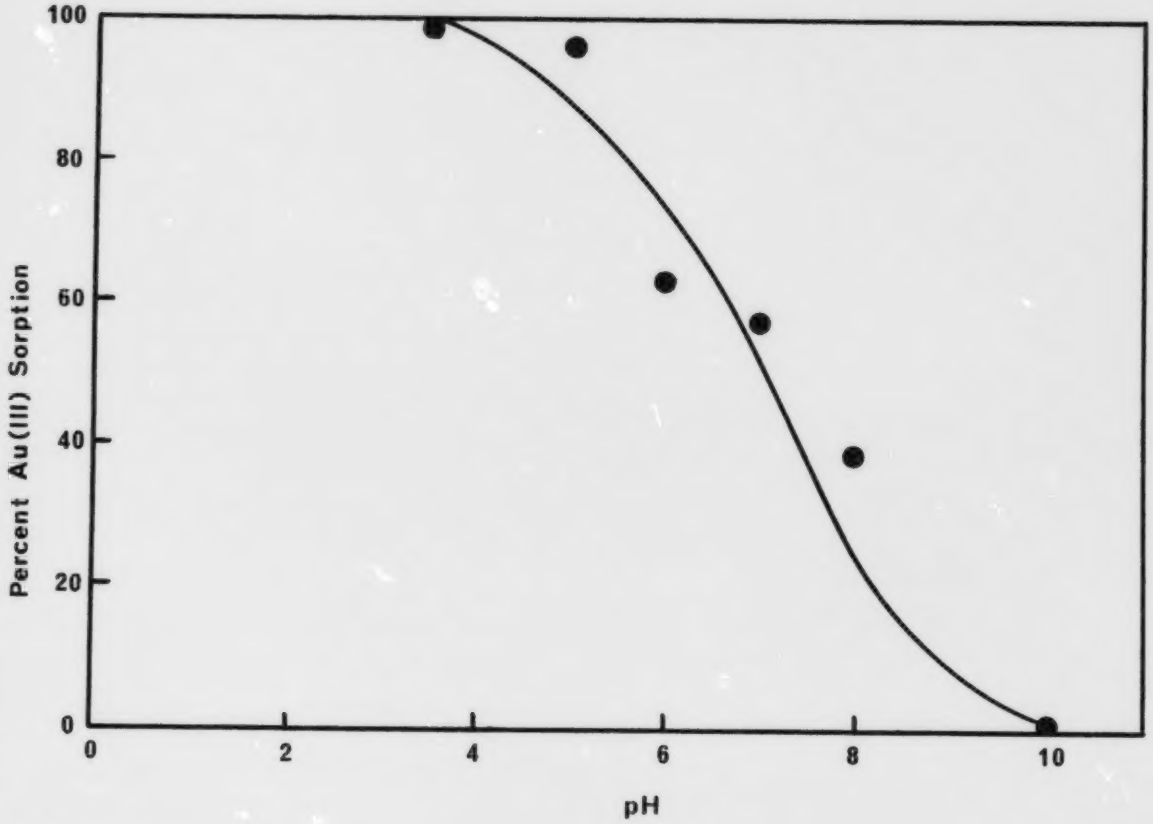

Fig. 6 Effect of pH on gold(III) ($AuCl^{-4}$) sorption on chitosan.

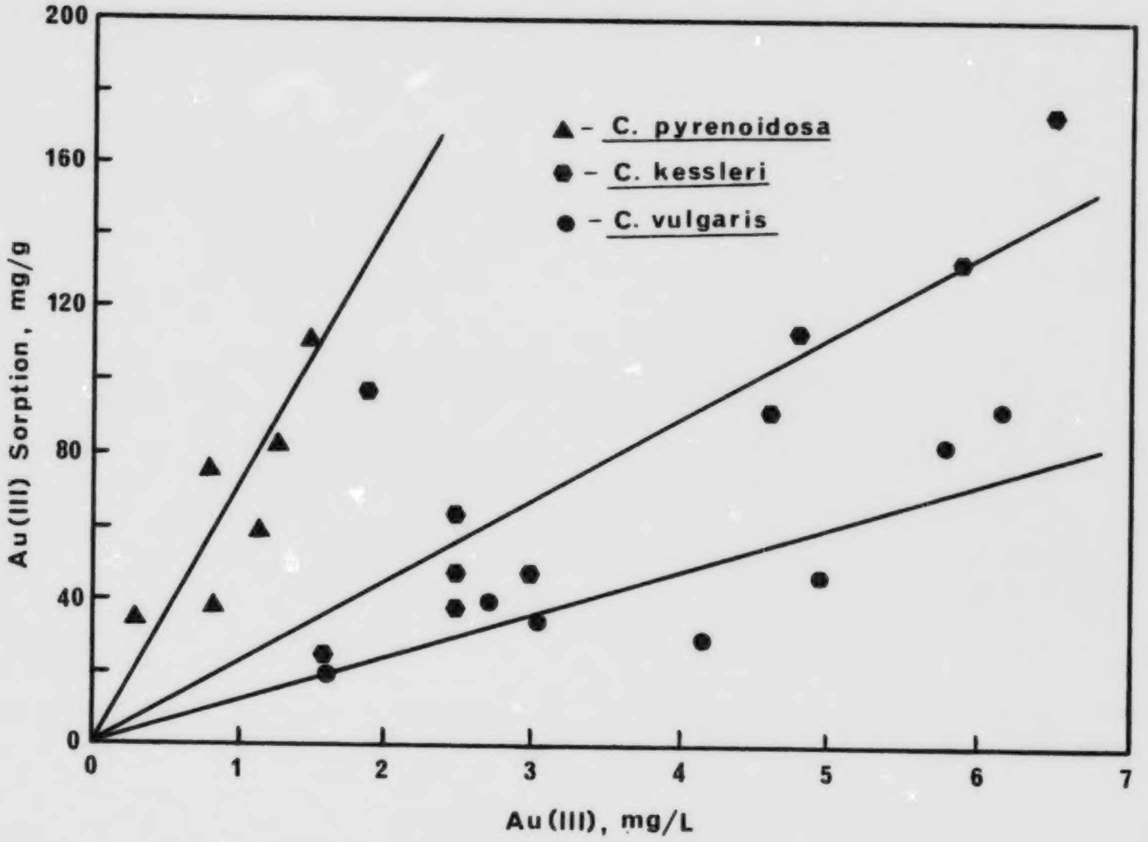

Fig. 7 Gold(III) ($AuCl^-_4$) sorption isotherm with algae.

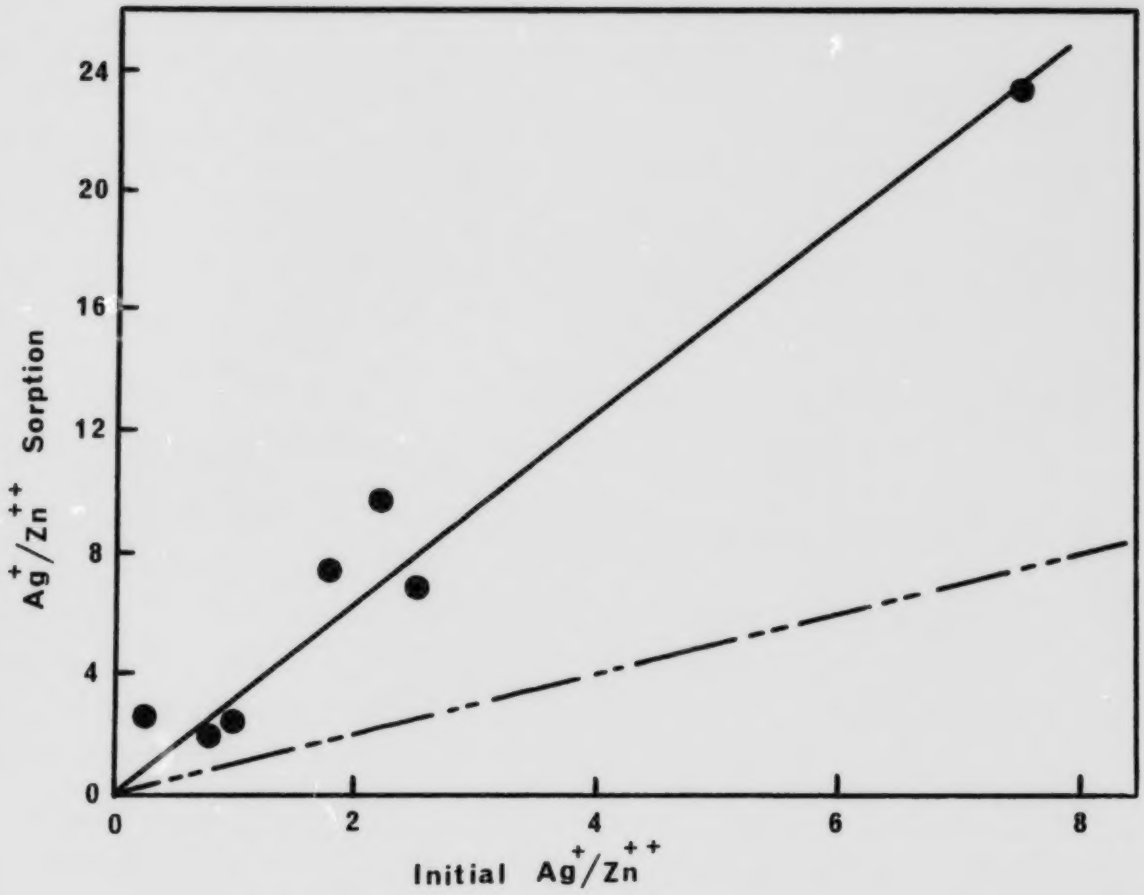

Fig. 8 Silver/zinc selectivity of chitosan.

BIOSORPTION OF METAL CONTAMINANTS USING IMMOBILIZED BIOMASS

by

T. H. Jeffers
Supervisory Chemical Engineer

C. R. Ferguson
Chemical Engineer

D. C. Seidel
Research Supervisor

Salt Lake City Research Center Bureau of Mines
U.S. Department of the Interior Salt Lake City, UT

ABSTRACT

The ability of microbial populations to sorb metals from dilute aqueous solutions is well documented. Although microorganisms have significant potential for decontaminating wastewaters, techniques and equipment suitable for bioassisted decontamination are limited. The Bureau of Mines has investigated the use of biomass immobilized in porous polysulfone beads for extracting toxic and heavy metals from dilute waste streams. The beads, designated as BIO-FIX beads, were fabricated from high-density polysulfone dissolved in dimethylformamide (DMF). Dried, thermally-killed biomass produced by algae, yeast, bacteria, and aquatic flora were blended into the polysulfone-DMF solution, and spherical beads were formed by injecting the mixture into water. Microscopic analysis revealed that the biomass was immobilized within the bead pores. BIO-FIX beads exhibited excellent handling characteristics and were utilized in stirred tanks, fixed-bed columns, and fluidized—bed columns to sorb metal contaminants from several wastewaters. Contaminants removed from the waters included arsenic, cadmium, copper, mercury, lead, manganese, and zinc. Laboratory tests indicated that the beads may be especially useful in treating dilute wastewaters containing metal concentrations up to about 15 mg/L. BIO-FIX beads are also well-adapted for use in secondary treatment or "polishing" of wastewaters. Elution of metal values from the beads was accomplished using dilute mineral acids. Laboratory tests indicated that the beads continued to extract metal ions after repeated loading-elution cycles.

BIOSORPTION DES CONTAMINANTS MÉTALLIQUES PAR LA BIOMASSE IMMOBILISÉE

T.H. Jeffers, C.R. Ferguson, et D.C. Seidel

Centre de Recherche Salt Lake City,
Bureau des Mines,

Département de l'intérieur des États-Unis,
Salt Lake City, UT É.-U.

RÉSUMÉ

La capacité des populations microbiennes à sorber les métaux contenus dans des solutions aqueuses est très bien connue. Même si les micro-organismes présentent un potentiel important pour la décontamination des eaux usées, les techniques et les appareils de décontamination par les micro-organismes sont limités. Le Bureau des Mines a étudié l'utilisation de biomasse immobilisée dans des billes de polysulfone poreuse afin d'extraire les métaux lourds et toxiques des eaux usées diluées. Les billes, appelées BIO-FIX, sont faites d'un polysulfone à haute densité dissout dans du diméthylformamide (DMF). La biomasse produite par les algues, les levures, les bactéries et la flore aquatique est tuée thermiquement, séchée et mélangée à la solution polysulfone-DMF. On forme des billes sphériques en injectant le mélange dans l'eau. L'analyse microscopique démontre que la biomasse est immobilisée dans les pores des billes. Les billes BIO-FIX sont faciles à manipuler et ont été utilisées dans des réservoirs à agitation, des colonnes à lit fixe et à lit fluidisé pour extraire les contaminants métalliques de plusieurs types d'eaux usées. Les contaminants extraits sont entre autres l'arsenic, le cadmium, le cuivre, le mercure, le plomb, le manganèse et le zinc. Les essais en laboratoire montrent que les billes sont particulièrement utiles pour traiter les eaux usées diluées contenant des concentrations de métal pouvant atteindre 15 mg/L. Elles sont aussi utiles pour le traitement secondaire des eaux usées. L'élution du métal contenu dans les billes est effectuée à l'aide d'acides minérales dilués. Les essais en laboratoire démontrent que les billes continuent d'extraire les ions métalliques même après plusieurs cycles de chargement et d'élution.

INTRODUCTION

The removal of toxic and heavy metal contaminants from aqueous waste streams is one of the most important environmental issues facing the United States today. Although this issue has been addressed for many years, effective treatment options are limited. Chemical precipitation, ion-exchange, reverse osmosis, and solvent extraction are the most commonly used procedures for removing metal ions from dilute aqueous streams (Rich, *et al*, 1987). However, these procedures have significant disadvantages such as incomplete metal removal, high reagent or energy requirements, and generation of toxic sludge or other waste products that require disposal. These disadvantages are particularly apparent at the low metal concentrations often encountered in wastewaters.

The search for new and innovative treatment technologies has focused attention on the metal binding capacities of various microorganisms. Yeast (Pryfogyle, *et al*, 1988), algae (Jennett, *et al*, 1982), bacteria (Norberg, *et al*, 1984), and various aquatic flora (Burton, et al, 1979; Ngo, *et al*, 1987) have been identified as organisms capable of sorbing toxic and heavy metals from dilute aqueous solutions. The mechanisms associated with metal sorption by microorganisms are complex and involve both extracellular and intracellular metal binding (Doyle, *et al*, 1980; Volesky, 1987). Extracellular metal accumulation has been reported as the more rapid mechanism and likely has the more significant role in metal sorption from wastewaters.

Although living microbial populations are effective sorbents for toxic and heavy metals, available processing systems are cumbersome. Living biosorption systems often require the addition of nutrients, and maintenance of a healthy microbial population may be difficult owing to the toxicity of the wastewaters processed. Recovery of the metal-laden microorganisms from solution is also troublesome due to liquid-solid separation problems.

Many species of yeast, algae, and other biomass sorb metals more effectively as a nonliving biomass (Tsezos, 1985; Kuyucak, *et al*, 1988), and their use eliminates the need to supply nutrients, as well as the problem of toxic shock. However, recovery of the metal-laden biomass is still cumbersome. Researchers have recognized that immobilizing nonliving biomass in a granular or polymeric matrix may improve biomass performance and facilitate separation of biomass from solution (Darnall, *et al*, 1986; Brierley, *et al*, 1987). This approach is currently being pursued by the Bureau of Mines. Porous polysulfone beads containing thermally-killed biomass were fabricated and utilized to remove metal contaminants from a variety of mining and mineral processing wastewaters. Preliminary tests indicated that beads, designated as BIO-FIX (an acronym for biomass-foam immobilized extractant), were readily prepared from commercially-available raw materials, were stable in strong acid and base solutions, and could be utilized in conventional hydrometallurgical processing equipment. Based on these initial results, additional studies were conducted to further define the performance of BIO-FIX beads. The objectives of this investigation were to demonstrate the potential of the beads for removing metal contaminants from representative wastewaters and to indicate factors that impacted the sorption and elution characteristics of the beads.

PREPARATION OF BIO-FIX BEADS

Identification of Bead Fabrication Parameters

BIO-FIX beads were prepared by dissolving high-density polysulfone in an organic solvent, blending dried biomass into the solvent, and injecting the mixture into water. Although durable beads were prepared using a wide range of fabrication parameters, studies were conducted to identify factors having the most significant effects on the chemical and physical characteristics of the beads. Variables investigated included the concentration of biomass in the beads, the polysulfone-to-solvent ratio used during bead preparation, the solvent type, the composition and temperature of the polymerizing media, and the bead curing time.

Only the biomass concentration and the polysulfone-to-solvent ratio had significant effects on bead performance. As expected, equilibrium metal sorptions increased as the biomass concentration increased. Quantitative data regarding this parameter are presented in the following section. The most significant effect of the polysulfone-to-solvent ratio was on the physical characteristics of the beads. Scanning electron microscope (SEM) analysis indicated that the average pore size, and hence porosity of the beads, increased as the ratio of polysulfone decreased. The increased porosity enhanced the metal sorption kinetics. Non-linear regression analysis indicated that a 40% increase in the rate of metal sorption was achieved when the polysulfone concentration used during bead preparation was decreased from 200 to 100 g/L of DMF. However, physical deterioration of beads prepared from solutions containing 75 g or less of polysulfone per liter of DMF was noted after several cycles. Also, problems were occasionally encountered during fabrication regarding sphericity and size uniformity with beads prepared from solutions containing 75 to 100 g polysulfone per liter of DMF. Thus, beads prepared from solutions containing 100 to 150 g/L polysulfone exhibited the most favorable combination of sorption kinetics and physical stability.

Effect of Biomass Concentration

Various amounts of dried, minus 100-mesh, blue-green algae (*Spirulina sp.*) were blended into a solution containing 120 g/L polysulfone dissolved in dimethylformamide (DMF). The blended mixture was injected into water, and the resulting beads were cured by stirring in water for 16 hr. The cured beads were contacted in a stirred tank for 24 hr with a wastewater containing 4.0 mg/L Mn and 0.06 mg/L Cd. As previously noted, the beads containing the greatest amounts of algal biomass extracted greater quantities of manganese and cadmium. However, the increase in metal sorption was not directly proportional to the biomass concentration. For example, beads containing 300 g biomass per liter of beads extracted only 1.5 times as much manganese and 1.8 times as much cadmium as beads containing 150 g biomass per liter.

Surface area analysis indicated that the porosity of the BIO-FIX beads decreased as the biomass concentration increased. Subsequent microscopic examination revealed that many of the interior pores were tightly packed with biomass at the higher concentrations. Apparently, the decreased porosity and tight biomass packing inhibited metal ion diffusion into the beads. Thus, metal extraction efficiencies were not directly proportional to biomass concentrations in the beads.

Standard Bead Fabrication Procedure

Based on results from the bead fabrication studies, a standard procedure was established for preparing BIO-FIX beads. Thermally-killed biomass was ground in a ring and cylinder pulverizer to minus 100 mesh and blended into a solution containing 100 to 150 g of polysulfone per liter of DMF. The biomass-polysulfone—DMF mixture was injected through a 2-mm nozzle into water, and durable, spherical beads were immediately formed. Bead size varied in response to the size of the injection nozzle and air pressure. Depending on the targeted application, beads ranging in size from minus 8 plus 12 mesh to minus 48 plus 100 mesh were produced. The beads were moderately agitated in water for 16 hr to allow organic solvent to leach out of the beads. The curing procedure also hardened the beads and ensured formation of a favorable pore structure. A cross-sectional photograph (24 X magnification) of a cured bead showing the porous nature of the bead interior is shown in Figure 1. The dark matter occupying several of the pores is immobilized algal biomass.

Selection of Biomass

The types of biomass studied in this investigation were selected following consultation with other researchers, literature searches, and sorption screening tests. Microorganisms reported as having metal sorption capabilities were obtained, and biomass was prepared by drying the material at 103° C for several hours. The biomass was then immobilized in polysulfone beads and contacted with wastewaters containing arsenic, cadmium, copper, zinc, and manganese. The screening tests were conducted in a stirred reactor using an aqueous-to-bead (A:B) volume ratio of 20:1. Biomass, which sorbed greater than 25% of one or more metal contaminants, was subjected to additional sorption tests. Based on their sorption potential and availability, a marine alga (*Ulva sp.*), a blue-green alga (*Spirulina sp.*), a yeast (*Saccharomyces cerevisiae*), common duckweed (*Lemna sp.*), xanthan and guar gums, and alginate (a carbohydrate polymer) were selected for further study. The objective of this investigation was to evaluate BIO-FIX bead performance, and no attempt was made to identify the most effective individual biomass material for site specific applications.

EXPERIMENTAL PROCEDURE

The handling characteristics of the beads containing immobilized biomass are similar to those of ion-exchange resins. Thus, conventional hydrometallurgical processing equipment such as stirred tanks, fixed-bed columns, and fluidized-bed columns were utilized for contacting the beads with wastewaters. All tests were conducted at ambient conditions.

Several wastewaters, typical of those emanating from mining and mineral processing operations, were studied in this investigation. Each of these waters is characterized by pH and contaminant levels in Table 1. The federally mandated National Drinking Water Standard (NDWS) (Anon., 1986) for each contaminant is also given. Although the individual contaminant levels in some of the waters were below NDWS prior to treatment, the waters were treated to demonstrate the effectiveness of the beads at very low metal concentrations. The waters include acid mine drainage waters and contaminated groundwaters from copper, gold, lead, and zinc operations. The waters also include those collected from sites addressed by the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) (Anon., 1980). This act, also known as "Superfund", resulted in the National Priorities List, which identifies over 1,000 of the most hazardous waste sites in the United States, including a number of mining and mineral processing sites.

EXPERIMENTAL RESULTS

All tests conducted during this investigation utilized actual wastewaters; no synthetic solutions or simulated waters were used. Because the waters varied considerably in metal concentration and pH, complete removal of all contaminants from each water was not attempted.

Metal Contaminant Concentration

Mining and mineral processing wastewaters contain concentrations of metal contaminants ranging from a few micrograms per liter up to several grams per liter. Thus, a major task of this investigation was to determine the range of metal concentrations applicable to treatment using BIO-FIX beads.

The effect of metal contaminant concentration on bead performance was evaluated using beads containing 250 g/L *Spirulina sp.* blue-green algae. The beads were contacted in a stirred tank with a pH 4.0 wastewater containing 32.5 mg/L Zn and 34.0 mg/L Mn. Minus 24– plus 48-mesh beads were used at A:B ratios of 1:1 to 100:1, and the contact time was 24 hr. Five commercially-available cationic ion-exchange resins were also contacted with the wastewater using identical conditions. The equilibrium loading isotherms generated from the test results for the *Spirulina sp.* beads and the ion-exchange resin exhibiting the greatest zinc and manganese loadings are shown in Figures 2 and 3. The results illustrate that the beads were considerably more effective in sorbing zinc and manganese at low metal concentrations. For example, a liter of the beads sorbed about 4 times as much zinc and manganese as the ion-exchange resin when the metal concentration was about 5 mg/L. The advantage enjoyed by the BIO-FIX beads was even more pronounced at metal concentrations near 1 mg/L. This sorption pattern did not reverse until the zinc and manganese concentrations increased to 20 mg/L. At these higher concentrations, the ion-exchange resin exhibited greater zinc and manganese loadings than the beads. Similar results were obtained with the other resins studied which indicated that BIO-FIX beads are most effective at metal concentrations up to about 15 mg/L.

A further example of the effectiveness of BIO-FIX beads at low contaminant levels was demonstrated by contacting a wastewater containing 45 μg/L Cd with beads containing 250 g/L *Spirulina sp.* algae. The beads and each of the five ion-exchange resins were contacted with the water for 24 hr in a stirred tank at an A:B ratio of 50:1. The ion-exchange resins extracted only 15 to 47% of the cadmium, and none of the effluents achieved the NDWS of 10 μg/L. In contrast, the BIO-FIX beads sorbed 90% of the cadmium, and satisfied the NDWS by reducing the effluent to 4.5 μg/L.

Metal Sorption Kinetics

The rate of metal sorption using BIO-FIX beads was also investigated. Beads containing immobilized duckweed (*Lemna sp.*), algae (*Spirulina sp.*), or xanthan gum biomass were contacted in stirred tanks and fluidized bed columns with several of the wastewaters identified in Table 1. Test results indicated that 70 to 90% of the equilibrium extraction was generally achieved in 30 min. Sorption continued at a much slower rate for the next several hours.

Cadmium and manganese sorption curves using beads containing 60 g/L duckweed for cadmium extraction and 150 g/L algae (*Ulva sp.*) for manganese extraction are illustrated in Figure 4. The beads sorbed 74% of the cadmium from an 18-μg/L wastewater in 30 min, and 82% of the cadmium in 60 min. The extraction after 2,880 min (48 hr) increased only slightly to 91%. Likewise, 62% of the manganese was extracted from a wastewater containing 4.0 mg/L Mn after 30 min. The extraction after 2880 min was 88%. This pattern of initial accelerated metal sorption followed by a much slower sorption phase was evident throughout the testing program and was independent of the aqueous metal concentration, the biomass type, the A:B ratio employed, or the type of contactor utilized.

Sorption of Calcium and Magnesium Ions

Many mining and mineral processing wastewaters contain appreciable amounts of calcium and magnesium ions. Since many ion-exchange resins readily sorb these ions, the capacity of the resins for toxic and heavy metals is decreased. The sorbed calcium and magnesium also interfere with elution and regeneration procedures. Unlike ion-exchange resins, BIO-FIX beads exhibit selectivity for heavy metal ions over calcium and magnesium. This selectivity was demonstrated in a three-column fixed-bed circuit utilizing beads containing 250 g/L blue-green algae (*Spirulina sp.*). Fifty bed volumes (BV) of a wastewater from an inactive zinc mining operation were passed through lead and scavenger columns of the beads each cycle. As two columns were loaded, the third column (loaded during a prior cycle) was eluted. Elution of metal values was accomplished using 1 M sulfuric acid. In four loading-elution cycles, over 99% of the zinc, cadmium, and manganese were removed from the wastewater. Calcium and magnesium ions were initially sorbed by the BIO-FIX beads but were readily displaced from the loading columns as the beads became saturated with heavy metal ions. As a result, only 9% of the calcium and 4% of the magnesium were extracted by the beads. This crowding effect was also observed in tests using duckweed or alginate BIO-FIX beads.

Secondary Treatment of Wastewaters Using BIO-FIX Beads

As discussed in the Introduction section, chemical precipitation is the most widely used process for removing metal contaminants from aqueous wastes. However, effluent concentrations meeting the NDWS are sometimes difficult to achieve using precipitation alone, and secondary treatment is necessary. Since BIO-FIX beads readily sorb metals from the near-neutral solutions resulting from precipitation processes, use of the beads as a secondary or "polishing" technique was investigated.

A complex wastewater from a Superfund site was subjected to chemical precipitation by adding a slurry of $CaCO_3$ until a final pH of 7.0 was reached. The pH was maintained for 4 hr, at which time the solution was filtered and sampled. The filtrate contained, in milligrams per liter, 1.3 Ag, 0.08 Mn, and 0.01 Pb. BIO-FIX beads at an A:B ratio of 30:1 containing 300 g/L *Ulva sp.* algae were then contacted with the filtrate in a stirred tank. After 1 hr of contact, 90% or greater of each targeted contaminant was removed from the filtrate, and the NDWS were met. The resulting metal ion concentrations, in milligrams per liter, were 0.011 Ag, 0.002 Mn, and 0.001 Pb. In comparative tests, a pH approaching 10 was required before precipitation alone achieved these levels. However, nearly twice as much reagent was consumed to reach this higher pH, and the volume of sludge generated was about twice as great at pH 10 as that generated at pH 7.

Elution and Regeneration of BIO-FIX Beads

Selection of Elution Reagents – Efficient removal of loaded metal values from the BIO-FIX beads was necessary to ensure their long-term use for repeated extraction-elution cycles. A review of current literature indicated that dilute mineral acids, ethylenediamine-tetraacetic acid (EDTA), and caustic solutions have been utilized to remove sorbed metal ions from microorganisms (Jennett, et al, 1982); therefore, these reagents were evaluated for eluting sorbed metals from the beads studied in this investigation.

EDTA and caustic solutions were only partially successful in eluting metal contaminants from BIO-FIX beads. For example, a solution containing 0.1 M EDTA stripped 75 to 85% of the zinc and 20 to 30% of the arsenic from algae (*Ulva sp.*) or alginate biomass beads. EDTA also eluted about one-half of the copper and manganese from yeast biomass beads. Solutions containing up to 0.3 M EDTA were studied, but no significant increase in metal elution was observed. Likewise, sodium carbonate and sodium hydroxide were effective eluates only in specific cases. For example, a 1 M-Na_2CO_3 solution removed >75% of the sorbed zinc from yeast and algae (*Ulva sp.*) beads, but <30% of the manganese and arsenic from alginate biomass beads.

Mineral acids, including sulfuric, nitric, and hydrochloric, were effective eluants. Acid solutions containing 0.1 to 0.5 M H_2SO_4, 0.05 to 0.1 M HNO_3, and 0.1 M HCl removed essentially all of the sorbed metals from beads containing yeast, duckweed, algae, alginates, or xanthan and guar gum biomass. The majority of elution occurred in the first 30 min of contact, and elution was essentially complete within 1 hr.

Concentration of Metal Values – Based on the elution study results, tests were conducted to demonstrate the potential of using BIO-FIX beads for concentrating metals extracted from wastewaters. A water containing 10.7 mg/L Zn and 0.062 mg/L Cd was passed through a three-column fixed-bed circuit containing 250 g/L algae (*Ulva sp.*) beads. Fifty bed volumes of water were passed through lead and scavenger load columns of the beads each cycle. While two columns were loaded, the third column was eluted of zinc and cadmium using 2 BV of 0.5 M H_2SO_4. The sulfuric acid eluate was recycled to each successive elution, and thus, was progressively enriched. After four cycles, the eluate contained 1,050 mg/L Zn and 6.42 mg/L Cd. This solution contained 98 times more zinc and 104 times more cadmium than originally contained in the wastewater solution. Essentially complete elution of zinc and cadmium were achieved during each elution cycle. Laboratory tests indicated that the acid eluate was amenable to further processing using conventional hydrometallurgical techniques such as ion exchange and solvent extraction.

Stability of BIO-FIX Beads – The chemical and physical stability of BIO-FIX beads are important considerations in determining their operating life for wastewater cleanup. Tests were therefore conducted to determine bead performance over several extraction-elution cycles. Beads containing duckweed (*Lemna sp.*) were contacted with a pH 3.8 mine drainage water containing 7.6 mg/L Zn and 4.2 mg/L Mn. In addition, xanthan gum and alginate biomass beads were contacted with mine drainage water and water containing 18 mg/L As and 1.7 mg/L Mn collected from the vicinity of a lead smelter. Elution of the duckweed beads was accomplished using 0.2 M H_2SO_4 while 0.1 M HNO_3 was used to elute the xanthan gum and alginate biomass beads. After 75 extraction-elution cycles, the duckweed beads showed no decrease in sorption or elution efficiency, and no physical deterioration of the beads was observed. Metal extraction efficiencies remained constant throughout the 75 cycles and averaged 92% for zinc and 90% for manganese. Likewise, excellent chemical and physical stability was observed during 44-cycle tests with beads containing xanthan gum or alginate. Metal extractions for zinc, manganese, and arsenic were, in percent, 85 to 95, 85 to 90, and 40 to 45, respectively. Visual observations indicated that stability of the beads for additional cycles was likely.

The excellent physical stability of BIO-FIX beads was also demonstrated by placing a 5-ft bed of beads in an ion-exchange column. Water was continually pumped downward through the beads for 30 days at a flow rate of 10 BV/hr. Physical analysis of the minus 14— plus 24-mesh beads after 30 days indicated that degradation due to attrition was nil.

SUMMARY AND CONCLUSIONS

BIO-FIX beads, containing thermally-killed biomass, were used to remove arsenic, cadmium, copper, mercury, lead, manganese, and zinc from several mining and mineral processing wastewaters. These toxic and heavy metals are among those most commonly encountered in the minerals processing industry. Effluents from the wastewater treatment frequently met the NDWS. The beads were fabricated from readily-available raw materials, were stable in acid and base solutions, and could be utilized in conventional hydrometallurgical equipment.

Sorption and elution tests with BIO-FIX beads containing immobilized algae, duckweed, yeast, xanthan gum, guar gum, or alginate biomass indicated the following:

1. The beads were most effective in treating wastewaters with metal concentrations ranging from 0.01 to about 15 mg/L.
2. Sorption of metal contaminants was fairly rapid during the first 30 min of contact; 70 to 90% of the equilibrium extraction was generally achieved in this time period.
3. The beads were effective as a secondary or "polishing" technique. For example, >90% of the remaining silver, manganese, and lead were sorbed from a chemical precipitation effluent.
4. Elution and regeneration of BIO-FIX beads was accomplished using sulfuric, nitric, or hydrochloric acid. As with the sorption process, elution of metal values from the loaded beads was fairly rapid. Complete elution was achieved within 1 hr. Recycling of the acid solutions yielded product eluates which were amenable to further processing using conventional hydrometallurgical techniques.
5. No decrease in sorption or elution efficiency was observed when BIO-FIX beads were recycled continuously for up to 75 extraction-elution cycles.

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TABLE 1

Metal Contaminant Analyses of Waste Solutions Investigated

Waste water	pH	Metal contaminant, mg/L					
		As	Cd	Cu	Hg	Mn	Zn
A	3.9	ND	ND	2.0	ND	52.0	241.0
B	13	3.5	ND	252	5.0	ND	2.9
C	3.8	ND	0.06	0.08	ND	4	8
D	6.7	18	ND	ND	ND	1.7	.1
E	6.4	1.5	0.02	ND	ND	.1	.02
F	6.9	ND	ND	ND	ND	1.8	2.8
NDWS	–	.05	.01	1	.002	.05	5

ND Not detected.

NDWS National Drinking Water Standard.

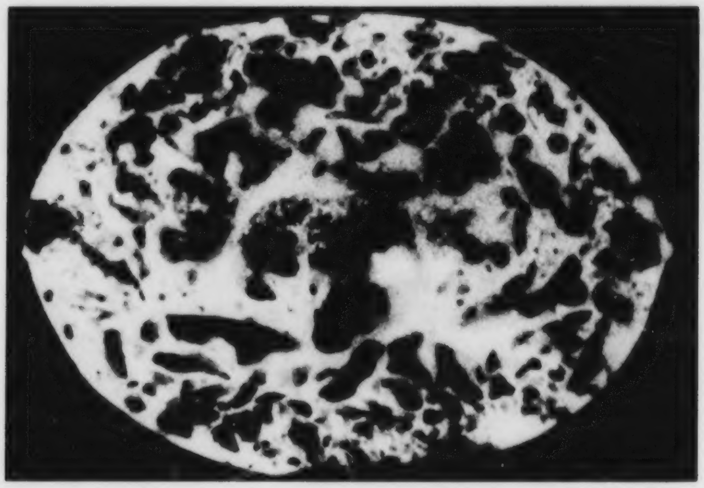

Fig. 1 Cross-sectional photograph of a polysulfone bead containing immobilized algal biomass.

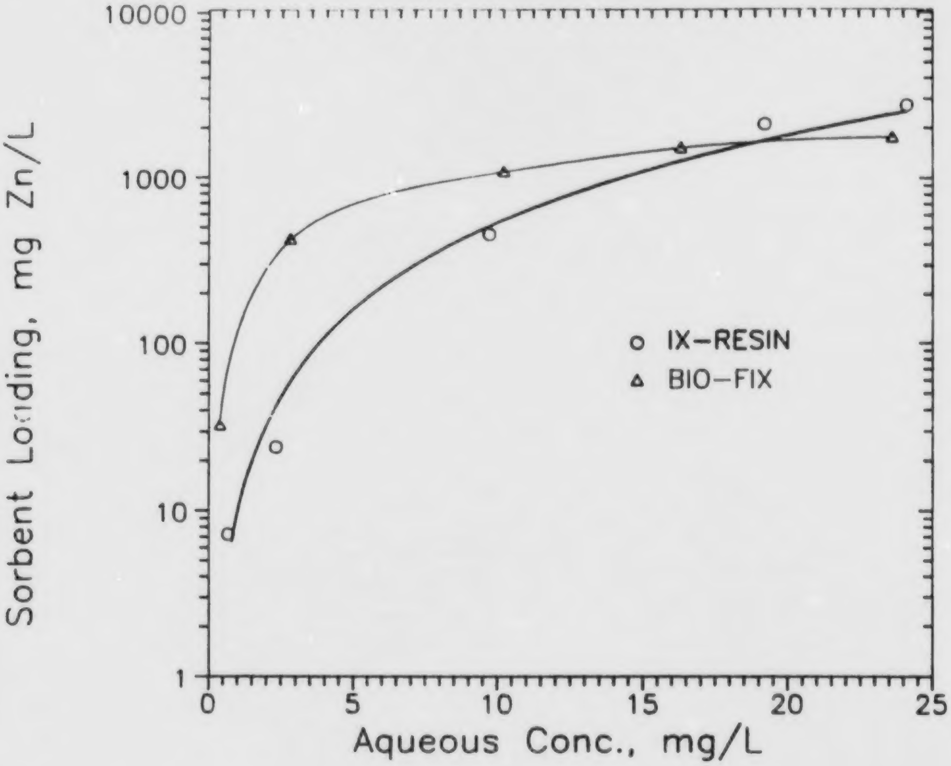

Fig. 2 Zinc equilibrium isotherm.

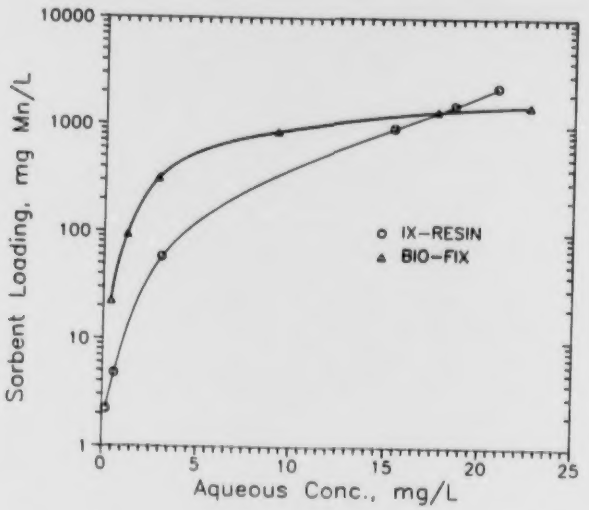

Fig. 3 Manganese equilibrium isotherm.

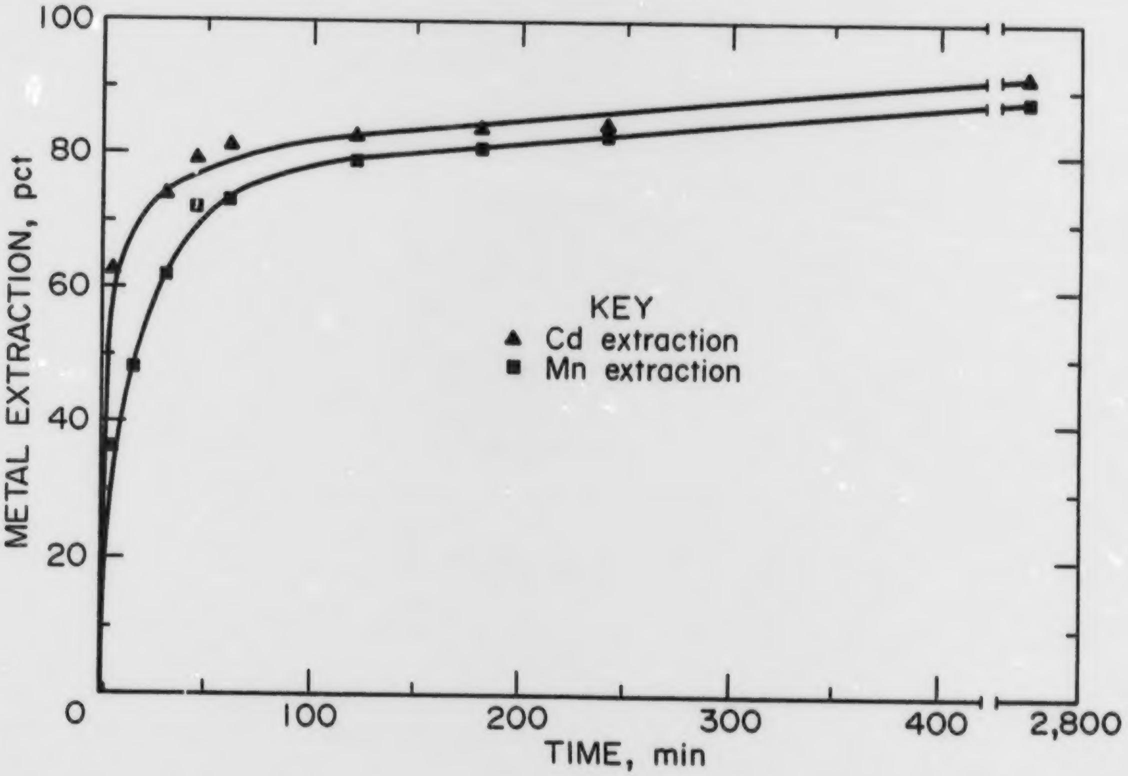

Fig. 4 Effect of contact time on metal sorption.

TREATMENT OF BIOMASSES FOR INCREASING BIOSORPTION ACTIVITY

F. Glombitza, U. Iske

Academy of Sciences of the GDR
Institute of Biotechnology Permoserstraße 15 Leipzig 7050

ABSTRACT

It was tried to characterize biomasses with regard to their ability of metal uptake and to collect parameters which influence the process and can be derived from the manner of cultivation and the state of the biomass.

Furthermore it was tried to enhance the sorption capacity by means of an aimed mechanical or chemical treatment. The results show a maximum of sorbed metals for the investigated microorganisms after a cultivation with N-limitation, an extraction with sodium lye or a mixture of methanol and chloroform or an ultrasonic treatment before and during the sorption process.

The electrophoretic mobility (EPM) in a an electric field was used for the characterization of the sorption property. It was possible to show a close connection between the EPM values and the metal amounts taken up by yeasts as well as bacteria.

TRAITEMENT DE LA BIOMASSE POUR AUGMENTER LA BIOSORPTION

F. Glombitza et U. Iske

Académie des Sciences de R.D.A.,
Institut de Biotechnologie,
Permoserstrasse 15, Leipzig 7050

RÉSUMÉ

On a tenté de classer les biomasses selon leur capacité de sorption du métal et d'établir les paramètres qui influent sur le procédé, paramètres qui dépendent du mode de culture et de l'état de la biomasse.

On a de plus tenté d'augmenter la capacité de sorption à l'aide d'un traitement mécanique ou chimique. Les résultats montrent que la sorption des métaux est maximale lorsque le micro-organisme à l'étude croît en fonction d'un facteur limite N et est extrait à l'aide d'une lessive de sodium ou d'un mélange de méthanol et de chloroforme ou lorsqu'il est traité aux ultrasons avant et pendant la sorption.

La mobilité électrophorétique dans un champ électrique a été utilisée pour caractériser la sorption. Il est possible de faire un rapprochement étroit entre les valeurs de mobilité électrophorétique et les quantités de métal extraites par les levures et les bactéries.

INTRODUCTION

At present it is attempted to use the ability of microorganisms to sorb metal ions from an aqueous medium due to their specific cell wall structure in technical processes for the metal separation from industrial waste waters (Brierly *et al.* 1986, Ringpfeil *et al.* 1987, Byerly *et al.* 1987, Tsezos 1988, Iske *et al.* 1988, Shumate II *et al.* 1985, Volesky, 1988).

The attainable maximum saturation concentrations and the conditions for their constant reproduction are highly important for the process manner. These saturation concentrations depend on the physical and chemical characteristics of the waste waters, e.g. pH value, temperature, concentration of the metal ions and state of the ions, and a number of biological characteristics as well. Iske *et al.* 1989, Gadd *et al.* 1988, Beveridge 1983, Diels *et al.*1988). The biological characteristics include all those parameters which can change the cell wall composition. These are, e.g. the kind of substrate, the limitation conditions and the residence time or the growth rate for a continuous fermentation process (Hübner *et al.* 1989).

The change of the cell wall may consist in a change of the number of functional groups or their states and thus the saturation concentration is influenced (Grimmecke *et al.* 1981, Brückner *et al.* 1981) (Table 1).

Furthermore, a treatment after cultivation for the partial or total removal of substances undesirable for the biosorption should give an increase of the amounts of metal taken up (Brierley *et al.* 1985, Heptinstall *et al.* 1970). The mechanical treatment for the enhancement of the sorption capacity is also based on an influence of ultrasonic waves on the cell wall (Bradshaw *et al.* 1987, Wagai *et al.* 1988).

TARGETS

Based on these connections it was tried:

- to characterize the biomasses concerning their capability of metal ion sorption in terms of their behaviour in an electric field for different pH-values by means of the electrophoretic mobility (EPM)
- to derive cultivation conditions which give a high loading capacity and
- to increase the loading capacity further by means of suitable mechanical and chemical treatment after cultivation

METHODS AND MATERIALS

Determination of the EPM

Yeasts and bacteria were placed in an electrical field and the mobility was determined at a current of 2 - 5 mA and different pH-values (Iske *et al.* 1989b). The EPM is the quotient of the velocity and the electric field strength. A Parmoquant 2 (Carl Zeiss, Jena) was used for the measurements.

Metal uptake-Biosorption

Biomasses were mixed with a metal ion containing solution in a stirred mixing device at known pH-value and temperature.

The alternation of the metal ion concentration was determined by an ion sensitive electrode and/or atom absorption spectroscopy.

Chemical treatment and extraction

50 g. dried biomass was mixed with 50 ml of chloroform, methanol or a mixture of the two at a ration of 2 : 1 at room temperature 3 times 30 min. After this, the solid phase was separated, dried and taken for the sorption.

The treatment with NaOH consists in mixing 50 g biomass with 1000 ml of a 3% - NaOH at 30°C for 30 minutes. The denaturated biomass was separated from the liquid, washed, dried and used for the sorption. The acid treatment was carried out with the strain MB 58 with gluconic acid. The biomass was used for the oxidation of glucose to gluconic acid according to the patent DD 218387 (Babel *et al.* 1983) and after reaching a gluconic acid concentration greater than 200g/l separated from the fermentation system and check in a sorption process.

Mechanical treatment

The mechanical treatment consists in an investigation of the ultrasonic and shear force influences. For this a liquid biomass suspension with a concentration of 2g/l as well as a biomass concentrate of 50g/l was taken.

The biomass suspension was treated with different ultrasonic generators, at different frequencies and for different times. Added to this was an after- or pretreatment, consisting in a homogenization by means of an ultra turrax stirrer. The ultrasonic treatment was also carried out during the sorption process.

Variation of the cultivation conditions

The microorganisms were cultivated in shaker flasks or in continuous stirred tank reactors.

The varied parameters were:

- the kind of substrate, an alteration between glucose and methanol or gelatine
- the dilution rate in the range from 0.05 h^{-1} to 0.5 h^{-1}
- the kind of limitation, C, N, O, P at a constant residence time of 12.5 h

Microorganisms

Gram negative methylotrophic microorganisms were used for the investigation in most cases. Some other strains were included in the investigations to complete the results.

Number and name of the strains used:

MB 53	neutrophilic, aerobic, methylotrophic bacteria, *Methylobacillus* sp.
MB 58	acidophilic, faultatively methylotrophic, aerobic bacteria *Acetobacter methanolicus*
MB 127	neutrophilic, methylotrophic, aerobic bacteria, methylamine utilizing, *Methylobacillus* sp.
MB 135	acidophilic, facultatively methylotrophic, aerobic bacteria, *Acetobacter methanolicus*
B 132	gelatinase forming, aerobic, neutrophilic bacteria, *Pseudomonas* sp.
EH 15	paraffin utilizing yeast - *Candida maltosa*

EH 55 paraffin utilizing yeast - *Yarrowia lipolytica*

EH 59 paraffin utilizing yeast - citric acid producing, *Yarrowia lipolytica*

RESULTS

Connection between EPM and metal uptake

The dependence of the EPM on the pH-value and the corresponding amounts of metal are demonstrated in Figure 1 for different microorganisms and metals. The number of free hydrogen ions decreases with increasing pH-value and hence the number of dissociated negative groups in the cell wall also increases. This is connected with an increase of the EPM. Simultaneously an enhancement of the fixed metal amounts taken up is shown in Figure 2 for different microorganisms.

A higher loading of the biomass with metal cations with increasing EPM is seen.

From this we can draw the conclusion that a characterization of the biomass concerning its ability to store metals on the cell envelopment is possible by means of the EPM.

CULTIVATING CONDITIONS

Residence time

The connection between the residence time and the metal uptake is presented in Table 2.

The microorganisms were cultivated with residence times in a range of 20 - 2.2 h according to growth rates of 0.05 - 0.45 h^{-1} with a constant limitation. The limitation was carbon limitation.

One part of the biomass was freeze dried after cultivation, the other part was used for biosorption as resting biomass. A slightly higher loading at high residence times is evident from these results as compared to the low residence times. Measurements of the EPM also showed nearly constant values and confirmed this fact.

Kind of substrate

The influence of the kind of substrate is presented in Table 3.

From the table is evident that a change of substrate and a shift to another substrate can give an increase (MB 53), a decrease (B 132) or a constant value (MB 58) of the sorbed metal amounts, if we start from glucose as a basic substrate.

Kind of limitation

The influence of the kind of limitation is different. A nitrogen limitation gives the highest metal amounts taken up, followed by carbon and oxygen limitation (Figure 3). Analyses of the fermentation broth showed a much higher content of polysaccharides with nitrogen limitation of the strain MB 53 than for the carbon limited process. This is due to the formation of an extracellular slime by MB 53 and nonoptimal fermentation conditions.

The slime is an acidic exopolysaccharide and consists of the monomers glucose, mannose, galactose and glucuronic acid. Its composition varies depending on the fermentation conditions (Table 4).

This slime is quite well suited for binding cations. The strain MB 135 shows a quite similar behaviour with regard to the limitation influence. Its tendency to slime production and the formation of an exopolysaccharide slime envelope however is of minor importance.

EXTRACTION PROCESSES

The influence of different extraction processes on the metal uptake and a comparison with the untreated biomasses is shown in Figure 4.

It is evident that the amounts taken up increase gently with the sodium lye treatment and an extraction with a mixture of chloroform and methanol. The metal uptake increases from 75 to 385 mg/g after a methanol/chloroform extraction and to 500 mg/g after sodium lye treatment for MB 127. The values increase from 25 mg/g biomass to 250 mg/g (extraction) and to 360 mg/g (sodium lye treatment) even for the yeast *Candida maltosa*.

These values do not contain any correction factors for the reduced biomass amounts after the treatment. Lipids are removed from the biomass in most cases and preferably in the extraction with the organic solutions (Biedermann *et al.* 1980) in contrast to a saponification and cleavage of the protein molecules taking place in the sodium lye treatment. The former treatment gives a quite good conservation of the shape of the cell, some constituents of which are in the aqueous medium, the latter, a nearly complete destruction of the cells and a preferable storage of the metal ions on the constituents of the cells (Figures 5, 6, 7).

Determinations of the EPM of the cell wall do not show any changes. This also demonstrates that the increase of the bound metal amounts is not connected with an enhancement of the cell wall negativity. The biomass reductions with extraction lie around 30% and for the sodium lye treatment around 50%. The corrected maximum amounts taken up still several times higher in comparison to the untreated microorganisms.

MECHANICAL TREATMENT OF THE BIOMASS

The important results are given in Table 5 and Figure 8. The highest metal cation concentrations taken up are reached with the treatment of the concentrate after a pretreatment and a continuation of the ultrasonic treatment during the sorption reaction.

The maximum value is about 130 mg/g bm in contrast to 45 mg of the untreated biomass. This means an enhancement of about 190% while treatments of the sorbens give only enhancement of about 30%. A treatment of the extracted biomass does not give significant enhancement of the metal uptake.

The dependence of the metal cations taken up on the power of the ultrasonic device at different frequencies shows a greater uptake at high frequencies and low powers than high powers and low frequencies.

The increase of the metal uptake can be explained by a partial slackening or destruction of the cell wall. The differences of the metal amounts taken up between a treatment during the sorption and a treatment without sorption indicate a partially irreversible change of the cell wall.

This can be confirmed by means of the desorption process. The total amount of cadmium of a loaded biomass is removed by means of a desorption process with inorganic acids. But in contrast to this a total desorption after an ultrasonic treatment is impossible.

CONCLUSIONS

The application of a biomass for the sorption of metals from an aqueous medium with the aim of storing a maximum amount of metal cations on the biomass needs extensive knowledge of the dependence of the sorption capacity on the cultivation conditions.

Nitrogen or substrate limitations are recommended in most cases as well as measurements of the EPM for testing the biomass ability. EPM measurements of the EPM for testing the biomass ability. EPM measurement can be made only in those cases where the sorption ability and its change are connected with an alteration of the electronegative state of the cell wall.

Mechanical and chemical treatments may increase the maximum metal amounts taken up considerably and are therefore to be taken into consideration. The enhancement of the sorption capacity is based on the use of cell constituents from the interior of the cells or break-up of the cell wall and a diffusion into deeper layers of the cell wall.

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Table 1

Change of the content of carbohydrates of EH 15 for different carbon sources and limitations of N.

CARBON-SOURCE	MANNAN	GLUCAN	TREHALOSE	GLYCOGEN	$\frac{\text{GLUCAN}}{\text{MANNAN}}$
GLUCOSE Ø	10.6	11.8	2.8	4.2	1.1
N-LIMITATION	13.1	19.6	6.0	12.8	1.5
PARAFFIN Ø	7.9	10.4	0.6	1.7	1.3
N-LIMITATION	8.1	13.7	1.8	5.8	1.7

Table 2

Influence of residence time and growth rate on the silver uptake for MB 53.

MB 53 (METHYLOBAC. SPEC.)
CARBON - LIMITATION

GROWTH RATE [h^{-1}]	0.05	0.1	0.16	0.2	0.25	0.35	0.40	0.45
RESIDENCE TIME [h]	20	20	6.25	5	4	2.85	2.5	2.22
BIOMASS - STATE: RESTING	45		40		39		40	40
FREEZE - DRIED		50		44	45	45		45

EPM — CONSTANT
SILVERCONCENTRATION (mg/g BM)

Table 3

Influence of the substrates on the silver sorption for different strains of microorganisms.

SUBSTRATE	MB53	MB58	B132
GLUCOSE	18.4	25.1	160.0
METHANOL	37.3	23.3	—
GELATINE	—	—	133.1

Table 4

Average composition of the microbial slime of MB 53.

CONSTITUENT	AMOUNT (%)		
	AVERAGE	MIN	MAX
CARBOHYDRATES (GLC)	55	44	69
URONIC ACID	25	22	30
HEXOSAMINE	<0.5		
MURAMIN	≤0.7		
ACETYL CONTENTS	2.7	2.3	3.1
CARBOHYDRATES			
FUCOSE	3	2.2	4.8
MANNOSE	20		
GALACTOSE	20	4	37
GLUCOSE	57	40	70

Table 5

Influence of the ultrasonic treatment in connection with pre- and after treatment on the cadmium uptake for MB 53.

MATERIAL	PRE AND AFTER TREATMENT	METAL UPTAKE			
		BEFORE TREATMENT	AFTER (mg/gBM)	INCREASE (mg)	%
BIOMASS 10' 8 MHZ 20W (2g/l)	WITHOUT	45	55	10	22
	+ SOAK HOMOGENIZATION		57	12	26
	+ TREATMENT DURING SORPTION		60	15	33
CONCENTRATE 10' 8 MHZ 20W (50g/l)	WITHOUT	45	75	30	66
	+ TREATMENT during sorption		88	43	95
	+ SOAK HOMOGENIZATION		132	87	193
EXTRACTED BIOMAS CONCENTRATE	+ SORPTION	55	71	16	29
	+ SOAK HOMOGENIZATION		74	19	35

MB 53 (METYLOBACILLUS SPEC.)
METAL: CADMIUM

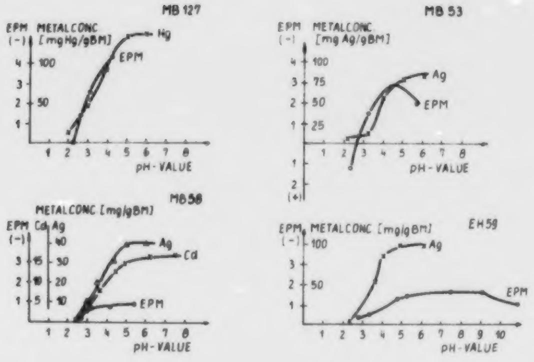

Fig. 1

Connection between EPM, pH-value and metal uptake

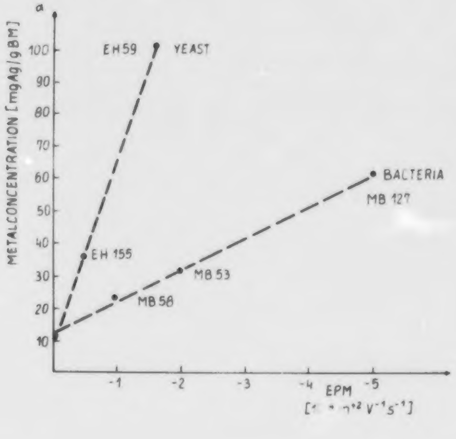

Fig. 2

Dependence of the metal uptake on the EPM for different strains of microorganisms.

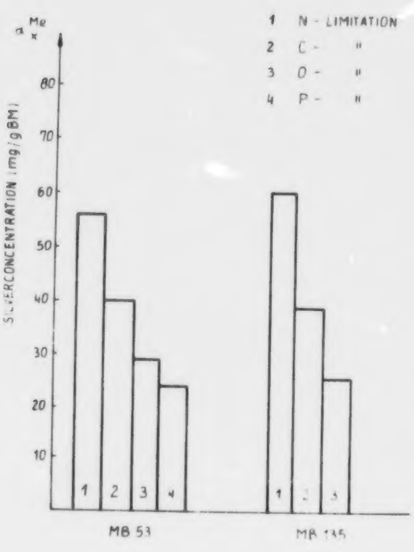

Fig. 3

Influence of the kind of limitation on the silver uptake for MB 53 and MB 135.

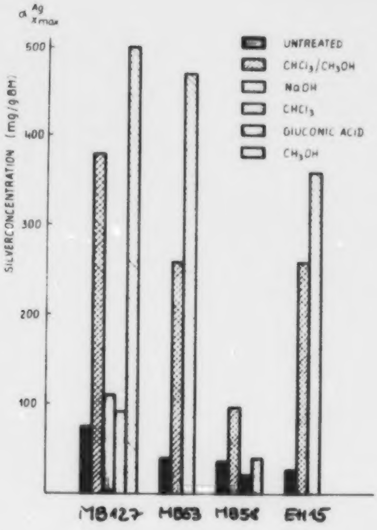

Fig. 4

Influence of the extraction manner on the silver uptake of the biomass.

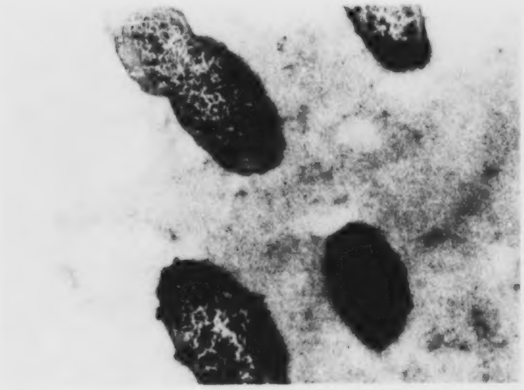

Fig. 5

Electron microscopic picture of MB 127 cells after silver sorption.

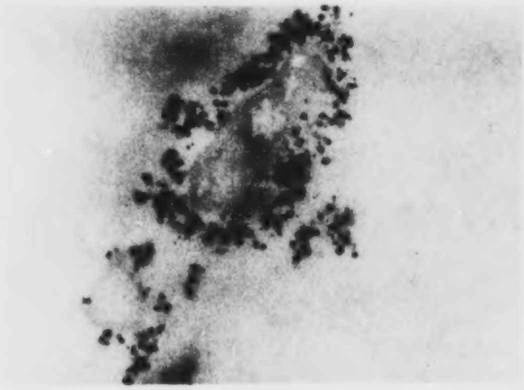

Fig. 6

Electron microscopic picture of MB 127 after silver sorption and treatment with $CHCl_3/CH_3OH$.

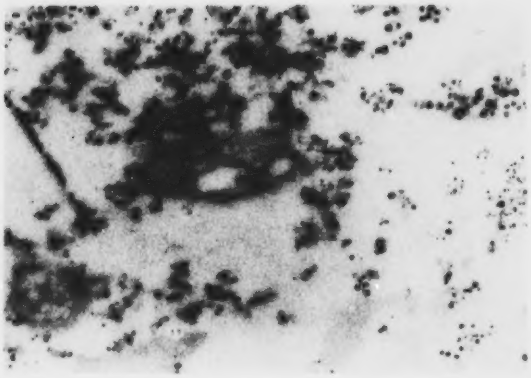

Fig. 7

Electron microscopic photo of MB 127 after silver sorption and treatment with NaOH.

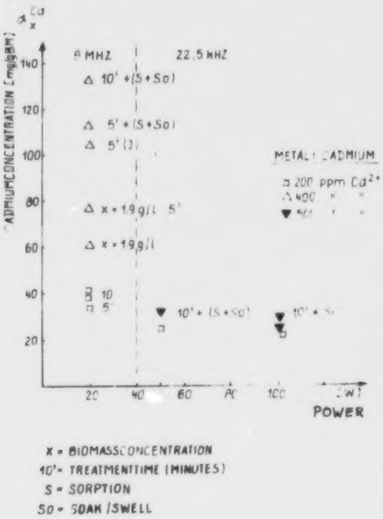

Fig. 8

Dependence of the sorbed cadmium amounts on the power and frequency of the ultrasonic treatment.

METAL RECOVERY FROM GEOTHERMAL WATERS AND GROUNDWATERS USING IMMOBILIZED ALGAE

Dennis W. Darnall, Robert M. McPherson and Jorge Gardea-Torresdey

Bio-recovery Systems, Inc. P.O. Box 3982 UPB Las Cruces, NM 88003, USA

ABSTRACT

The cell walls of a variety of microorganisms have high affinity for heavy metal ions. Metal ions are known to bind through nitrogen, sulfur and oxygen donors from cell wall biopolymers. The propensity of transition metal ions to form covalent bonds, as opposed to ions such as calcium, magnesium, sodium and potassium which tend to form ionic bonds, makes biological materials particularly useful for selectively recovering very dilute concentrations (ppb) of heavy metals from waters containing high concentrations of alkali and alkaline earth metal ions.

Bio-recovery Systems has developed a new preparation of immobilized algae (AlgaSORB® II) which is capable of selectively recovering precious metals from geothermal waters at well head temperatures. AlgaSORB® II has selectively recovered ppb levels of metals such as gold, silver and platinum from geothermal waters that have high concentration of salts (up to 260,000 ppm).

AlgaSORB® II is also particularly effective in recovering base metals from contaminated groundwaters. Low ppm levels of metal ions such as copper, mercury or cadmium are reduced in concentration to low ppb levels by treatment with AlgaSORB® II.

RÉCUPÉRATION DE MÉTAUX DE SOURCES GÉOTHERMALES ET D'EAUX SOUTERRAINES À L'AIDE D'ALGUES IMMOBILISÉES

D.W. Darnall, R.M. McPherson et J.G. Torresdey

Bio-recovery Systems Inc.
Las Cruces, NM É.-U.

RÉSUMÉ

Les parois cellulaires de plusieurs micro-organismes ont une grande affinité pour les ions de métaux lourds. Les ions métalliques se lient à une variété d'ions de métaux lourds à l'aide du nitrate, du soufre et de l'oxygène des parois cellulaires des biopolymères. La tendance des ions métalliques de transition à former des liens covalents, contrairement aux ions comme le calcium, le magnésium, le sodium et le potassium qui forment des liens ioniques, rendent le matériel biologique particulièrement utile pour la récupération sélective de concentrations très faibles (ppb) de métaux lourds contenus dans les eaux à forte concentration d'alcalins et d'ions métalliques alcalino-terreux.

Bio-recovery Systems a mis au point une nouvelle préparation d'algues immobilisées (AlgaSORB II) qui permet la récupération sélective des métaux précieux des eaux géothermiques à la température de tête des puits. AlgaSORB II a récupérée des métaux comme l'or, l'argent et le platine à des concentrations de l'ordre du ppb dans des sources géothermiques à forte concentration de sels (jusqu'à 260,000 ppm).

AlgaSORB II est particulièrement efficace pour récupérer les métaux alcalins des eaux souterraines contaminées. Les concentrations d'ions métalliques comme le cuivre, le mercure et le cadmium de l'ordre du ppm sont réduites à quelques ppb après traitement avec AlgaSORB II.

INTRODUCTION

Over the past few years a number of investigators have taken advantage of the unique metal ion binding capacity of the cell walls of microorganisms and have developed biomass materials which are either close to commercialization or are currently being commercialized (Darnall, 1988, 1989; Brierley *et al.* 1988; Kuyucak and Volesky, 1988; Tsezos, 1988). The chemical functional groups in biopolymers of the microorganisms cell walls provide excellent, very high affinity metal chelation sites. Thus, transition metal ions can often be scavenged from solutions which are very dilute in metal ions and which have a high concentration of other ionic components.

Darnall *et al.*, (1988) and Greene and Darnall (1988) reported that binding of many transition metal ions to the algae *Spirulina* and *Chlorella* is endothermic. These studies indicated that between the temperature range of 0°C and 60°C, the binding of gold(III) to *Spirulina* increased by nearly an order of magnitude. Other work has suggested that precious metals are more strongly bound to algal biomasses than any other transition metal ion studied (Darnall *et al.*, 1989; Greene *et al.*, 1989).

Several reports have appeared indicating that several geothermal fields containing waters from 150°C to 300°C contain small amounts of dissolved, ionic precious metals (Brown, 1986 and references therein). The endothermic nature of gold binding to algae, coupled with the high selectivity of binding of precious metals (in the presence of base metals) to algae, thus suggests that algal biomasses might be used to scavenge precious metals from geothermal waters if the biomass could be stabilized to withstand high temperatures and pressures. Herein we report on the preparation of a new biomass that has been stabilized to withstand temperatures as high as 260°. This new proprietary biomass has particularly good precious metal binding properties at elevated temperatures as well as at ambient temperatures and appears to be suitable for recovering transition metal ions in the ppb range from polluted groundwaters.

METHODS AND MATERIALS

A proprietary biomass-based metal binding material (AlgaSORB® II) was prepared from *Chlorella vulgaris* (Greene *et al.*, 1989). Beads of AlgaSORB® II which were sized to approximately 1 mm diameter were used for all studies.

High temperature metal binding studies were conducted in teflon-lined acid digestion bombs (Model 4749) obtained from the Parr Instrument Co., Moline, Illinois.

Metal ion analyses were conducted using flame or graphite furnace atomic absorption as previously described (Darnall *et al.*, 1986a). Precious metal-containing solutions were prepared from the following metal salts:$HAuCl_4$, $PtCl_4$ and $AgNO_3$.

RESULTS AND DISCUSSION

GEOTHERMAL WATERS.

An algal biomass (AlgaSORB®) immobilized in silica gel (Darnall *et al.*, 1986B) was initially tested for stability at elevated temperatures. This was done by placing some AlgaSORB® in an aqueous solution inside a Parr bomb, sealing the bomb and subsequently placing the bomb inside an oven at 190°C for four hours. Upon cooling the bomb, examination of the AlgaSORB® showed that it had completely decomposed. Subsequent incubations of AlgaSORB® at 190°C in water saturated with silica showed that massive decomposition of the silica gel immobilized algae occurred. Thus, efforts were initiated to stabilize a biomass for use at high temperatures. These efforts resulted in a new proprietary method of immobilization of algae (Greene *et al.*, 1989) which results in a biomass which is stable for at least six weeks (the longest time tested) at temperatures as high as 260°C (the highest temperature tested). This material, designated AlgaSORB® II, was then tested for precious metal ion binding.

Precious metal binding to AlgaSORB® II was first tested in Parr bombs at elevated temperatures in the laboratory. It was found necessary to have a completely teflon-lined system to avoid plate-out of precious metals on the stainless steel bomb at elevated temperatures. The bomb interior is shown in Figure 1. The teflon insert, which was placed inside the bomb, was designed so that the solution containing precious metals could be introduced into the bottom of the chamber. A teflon screen supported by teflon snap rings was designed so that AlgaSORB® could be placed upon the screen out of initial contact with the fluid. The insert, charged with both AlgaSORB® and a precious metal-containing solution, was then placed inside the bomb, the bomb sealed, and it was placed inside an oven at 190°C. After allowing four hours for the bomb and its contents to come to temperature equilibrium, the oven was opened and the bomb was inverted. This allowed the hot, metal-containing solution to run through the teflon screen and to come into contact with AlgaSORB® II. The oven was then closed and the bomb was allowed to incubate for 12 hours at 190°C. At the end of the incubation time the bomb was removed from the oven and immediately re-inverted so that the fluid was separated from the AlgaSORB®. After the contents of the bomb were cooled, the bomb was opened, the solution was analyzed for precious metal and the AlgaSORB® was stripped of metal for analysis.

The above procedure insured that the metal-binding experiments were done at 190°C and that complications due to metal ion binding at lower temperatures (as the bomb was being heated or cooled) were not side-by-side with the metal binding experiments.

Initial gold-binding studies were conducted with tetrachloroaurate(III). A solution containing 100 ppb (5.1×10^{-7} M) of gold as the tetrachloroaurate(III) complex in deionized water was incubated as described above with AlgaSORB®. After the contents of the bomb were cooled and analyzed by graphite furnace atomic absorption analysis, the data shown in Table 1 were obtained. Analysis showed no gold was present in the solution after contact with AlgaSORB® II at 190°C. AlgaSORB-free tests showed that the gold remained in the aqueous phase after the bomb heating.

Further testing of gold binding to AlgaSORB® at 190° was done in a matrix of geothermal water. This was done in order to see if a solution with a relatively high total dissolved solid content (3500 ppm) would interfere with gold binding. The geothermal water, initially at 165°C in the geothermal well, was collected and cooled. Upon cooling of the waters a precipitate of silica containing some base metals was separated by filtration. While the loss of some minerals in the precipitate changed the chemical composition slightly, these waters were nevertheless used for initial laboratory testing of gold binding. The filtered geothermal waters were then spiked with gold (100 ppb as $AuCl_4^-$) and bomb binding studies were conducted as described above at 190°C (Table 1). Analysis showed that approximately 90 percent of the gold was recovered on the AlgaSORB® even after the AlgaSORB® was taken through 14 separate loading cycles. After the 14th loading cycle AlgaSORB® had adsorbed gold to an extent of about 1.2 weight percent. Experiments were terminated after 14 gold-loading cycles even though no significant decrease of gold binding was observed.

The same type of metal-loading experiments at 190°C were done for silver(I) and platinum(IV). Table 1 shows results of these experiments. Silver at ppm levels was essentially completely bound whether from a matrix of deionized water or a solution of saturated sodium chloride (about 25% brine). By the same token, ppb levels of platinum were completely bound by AlgaSORB® from matrices of deionized water and saturated sodium chloride.

POLLUTED GROUNDWATERS

Removal of Cadmium from Waters at a Superfund Site.

Officials from U.S. EPA Region II arranged to supply samples from a well at a Superfund site in New Jersey, the Waldick Aerospace Devices site. These waters contained, among other things, cadmium at a level of 0.13 mg/L. The waters, at a pH of 6.0-7.1, also contained 0.66 mg/L of a halogenated hydrocarbon, tetrachloroethylene, as well as other organics.

A column containing AlgaSORB® (0.7 cm ID x 13 cm high) was prepared, and the Waldick Aerospace waters were passed through the column. Five milliliter fractions of water exiting the column were collected until 500 mL (100-bed volumes) of Waldick waters were passed through the column at a flow rate of one-sixth of a bed volume per minute (total bed volume was 5.0 mL). Each fraction of effluent was analyzed for cadmium using graphite furnace atomic absorption spectrometry. All effluent fractions showed that cadmium concentration was near or below 0.001 mg/L through the passage of the 100-bed volumes of the cadmium- containing solution. Because the experiment was stopped after the passage of 100-bed volumes through the column, it is not possible to state explicitly what volume of solution could be treated before cadmium breakthrough occurred. However, experience has shown that if a test material is capable of treating at least 100-bed volumes, it will be economically feasible to use the material. The essential point is that the AlgsSORB removed cadmium well below these levels which are allowed in drinking water. The current drinking water limits for cadmium stand at 0.005 mg/L.

Once 100-bed volumes of the cadmium-containing solution had passed through the AlgaSORB®-containing column, cadmium was stripped from the column by passing 0.15M H_2SO_4 through the column. Analysis of the column effluents showed that nearly 90 percent of the cadmium was stripped from the column with the passage of two-bed volumes of sulfuric acid through the column. Most of the remainder of the cadmium appeared in the next two-bed volumes. Mass balance calculations showed that, within experimental error, all of the bound-cadmium was stripped from the column.

Removal of Copper from Contaminated Groundwaters Containing Halogenated Hydrocarbons.

Ground waters obtained from a site contaminated with copper, trichloroethylene and dichloroethylene were tested for coppery recovery using AlgsSORB®. These waters contained a total dissolved solid content (TDS) of nearly 2000 ppm and had a total calcium and magnesium content of approximately 300 ppm. Past experience had shown that ion exchange resins were not effective in treating these waters for copper removal because of i) the high mineral content and ii) the propensity of the resins to become clogged with the organics in these waters. However, experiments showed that 400-bed volumes of the copper containing waters could be passed through a column (0.7 cm ID x 13 cm high) containing AlgaSORB® without effluents from the column containing more than 0.01 ppm of copper. The experiments were stopped with the passage of 400-bed volumes, so undoubtedly larger volumes of waters could have been treated before unacceptable levels of copper appeared in the effluents.

After 400-bed volumes had been passed through the AlgaSORB® column, the bound copper was, within experimental error, completely stripped from the column by the passage of 0.5M H_2SO_4 through the column. Again, as with the cadmium stripping, the copper was almost completely stripped within the first few bed volumes. Once stripped of copper, the column was ready to reuse.

Removal of Mercury from Contaminated Groundwaters

Bio-recovery was provided with water samples from a mercury-contaminated groundwater site. The site had been contaminated with mercury years ago through the process used to manufacture chlorine from seawater. The groundwaters contained 2-3 ppm of mercury (both inorganic and organic mercury), had a total dissolved solid content of 7,200 mg/L and contained over 900 mg/L of calcium and magnesium. Passage of over 300 bed-volumes of these mercury-containing waters through an AlgaSORB® column (0.7 cm ID x 13 cm high) resulted in effluents which ranged in mercury content as determined by analysis using cold vapour generation and atomic absorption spectrometry.

CONCLUSION

It is clear from the results shown in Table 1 that AlgaSORB® II is capable of binding very small amounts (ppm and ppb levels) of precious metals at elevated temperatures and pressures. Thus, the possibility exists for using AlgaSORB® to recover precious metals from naturally occurring geothermal waters which may contain low concentration of the precious metals. Particularly attractive would be those geothermal waters which are currently being used to generate electricity. While the concentrations of precious metals in geothermal waters is thought to be low (Brown, 1986), the very high flow rates at some of these geothermal electric plants indicates that significant quantities of precious metals may be available for recovery.

The AlgaSORB® II developed for use at very high temperatures also has high affinity for base metals at ambient temperatures. Thus, the use of AlgaSORB® II for treatment of metal-contaminated groundwaters appears to be feasible. Bio-recovery Systems is currently field testing AlgaSORB® for mercury removal from a contaminated groundwater under a Superfund Innovative Technology Evaluation (SITE) contract with the U.S. Environmental Protection Agency.

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Table 1

Gold, Silver and Platinum Recovery by AlgaSORB® at 190°C.[1]

Complex	Initial Concentration[2] (ppb)	Final Concentration[3] (ppb)	Aqueous Matrix
$AuCl_4^-$	100	ND[4]	DeionizedWater
$AuCl_4^-$	100	9	Geothermal Water
$AuCl_4^-$	100	11[5]	Geothermal Water
$AgNO_3$	3000	7	Deionized Water
$AgCl_2^-$	2500	ND[4]	Saturated NaCl
$PtCl_4$	46	ND[4]	Deionized Water
$PtCl_6^{-2}$	110	ND[4]	Saturated NaCl

[1] Experimental details of gold, silver and platinum binding to AlgaSORB® are given in text.

[2] Refers to metal ion concentration prior to contact with AlgaSORB®.

[3] Refers to metal ion concentration after contact with AlgaSORB®.

[4] ND – not detected.

[5] After 14th cycle of gold loading. See text for details.

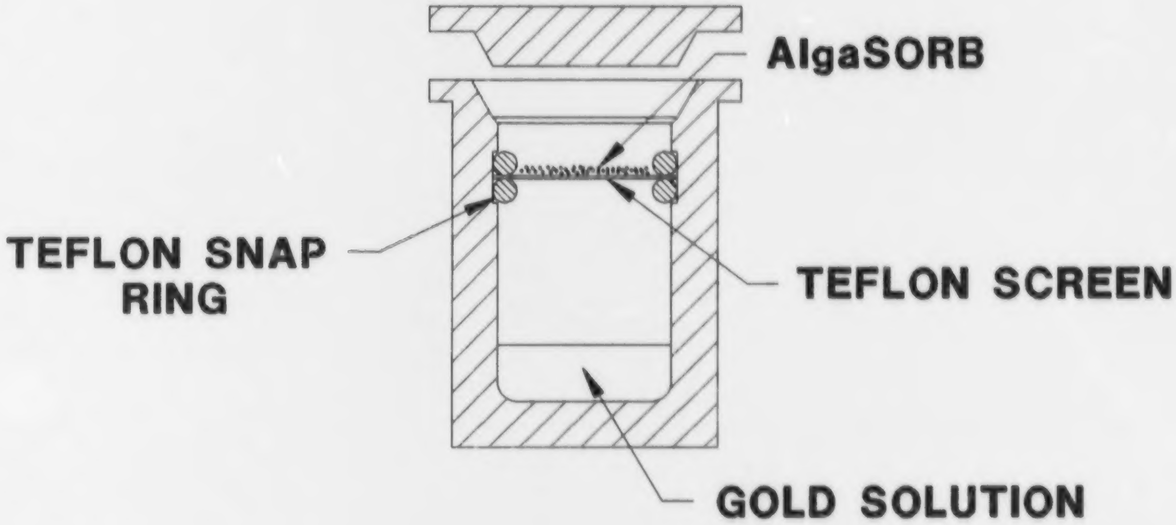
AlgaSORB
TEFLON SNAP RING
TEFLON SCREEN
GOLD SOLUTION

Fig. 1 Teflon Insert for Parr Acid Digestion Bombs. The teflon insert was modified as shown in order to examine metal ion binding at elevated temperatures. See text for details of operations.

BEHAVIOR OF IRON AND MANGANESE IN THE SEDIMENT OF A WETLAND SUBJECTED TO ACIDIC MINE DRAINAGE

William J. Tarutis, Jr. and Richard F. Unz

Environmental Pollution Control Program and Department of Civil Engineering
The Pennsylvania State University, University Park, PA 16802 U.S.A.

ABSTRACT

The behaviour of iron and manganese in a natural wetland receiving acidic mine drainage was characterized in order to determine the potential for long-term retention of these metals in the sediments. Five metal fractions sensitive to changes in environmental conditions and the possible association of iron and manganese with organic carbon and sulfur were investigated. Locations of metal retention and release were evaluated using multivariate statistical techniques. Concentration-depth profiles revealed differences in both the horizontal and vertical distributions of all parameters measured. The aeorbic zone was described by an abundance of iron and manganese oxides, organic carbon, and sulfur in the sediment and virtually no dissolved iron and manganese in the interstitial water. The anaerobic zone was relatively low in iron and manganese oxides with a concomitant increase in dissolved iron and manganese in the interstitial water. Metal sulfides were low in the anaerobic zone. High carbon to sulfur ratios suggested that bacterial sulfate reduction was of minor importance in metal removal, presumably, due to insufficient available organic matter. Adequate decomposable organic substrates in the anaerobic zone would likely enhance the formation of iron sulfides and provide a permanent sink for long-term metals retention.

COMPORTEMENT DU MANGANÈSE ET DU FER DANS LES MARAIS SALANTS AFFECTÉS PAR LES RÉSIDUS ACIDES DES MINES

William J. Tarutis, Jr. et Richard F. Unz

Environmental Pollution Control Program and Department of Civil Engineering
Pennsylvania State University, University Park, PA 16802 U.S.A.

RÉSUMÉ

Le comportement du fer et du manganèse dans les marais où s'écoulent les résidus acides des mines a été étudié afin de déterminer le potentiel de rétention à long terme de ces métaux. On a étudié cinq fractions métalliques sensibles aux changements des conditions environnementales ainsi que la possibilité que le fer et le manganèse s'associent au carbone et au soufre organique. Les emplacements de rétention et de libération des métaux ont éte évalués à l'aide de techniques statistiques à plusieurs variables. Les profils de concentration en fonction de la profondeur ont révélé des différences dans les distributions horizontales et verticales de tous les paramètres mesurés. Dans la zone aérobie, on trouve des oxydes de fer et de manganèse, du carbone organique et du soufre en abondance dans les sédiments, mais très peu de fer et de manganèse dissout dans l'eau interstitielle. La zone anaérobie est relativement pauvre en oxydes de fer et de manganèse, mais la concentration de fer et de manganèse dissout dans l'eau interstitielle augmente. Les sulfites métalliques sont rares dans la zone anaérobie. Le rapport élevée carbone/soufre indique que la réduction bactérienne du soufre est peu importante dans l'extraction des métaux, probablement en raison de la faible quantité de matières organiques disponibles. Une quantité suffisante de substrat organique décomposable dans la zone anaérobie favoriserait probablement la formation de sulfures de fer et formerait un bassin permanent de rétention à long terme des métaux.

INTRODUCTION

The drainage from abandoned mines typically are moderately to very acidic, very hard, and have high concentrations of metallic cations associated with them. If left untreated, these drainages can have significant impacts on the environment. Physical-chemical treatment of acidic mine drainage (AMD) is the usual means for meeting effluent quality limitations; however, such treatment is often technologically difficult and expensive. Recently, researchers have investigated biological treatment of AMD using wetlands (Unz and Dietz, 1986). The capability of wetlands to effect long-term removal and retention of these metals is of prime importance in their use as a pollution abatement tool. Ultimately, the long-term sink for metals such as iron and manganese in wetlands is the sediments.

The purpose of this research was to describe the behaviour of sedimentary iron and manganese in an AMD wetland system in order to determine if wetlands receiving AMD adequately remove and retain these metals in the long term. This research was primarily concerned with physicochemical interactions between these metals and their associated solid and aqueous phases.

MATERIALS AND METHODS

Description of Study Area

An abandoned mine site located near PA Routes 28 and 861 In New Bethlehem, PA, Clarion County (41° 02' 35"N, 79° 21' 25"W) was chosen to study the ability of a wetland to remove iron and manganese from two mine drainage seeps: Seep C (flow 10-15 m^3/day, pH ~ 7) and Seep S (flow 0.1-2m^3/day, pH ~ 5.5). The study area had an abundance of diverse vegetation during the spring and summer months consisting largely of cattails (*Typha latifolia*) and *Equisetum* sp. as well as a mixture of dicot, grass and sedge species. Mosses and algae were present also, but to a lesser extent.

Sampling Procedures

Sediment collection. Sediment samples were collected during October 1987 in duplicate at the influent and effluent of Seep C and in triplicate at the influent, mid-point, and effluent of Seep S using a one-inch diameter brass tube corer. Each sampling station was separated by a distance of about 7 meters. The cores were extruded in the field and transported on ice to the laboratory.

Interstitial water collection. Interstitial water samples were obtained *in situ* during August 1988 using an interstitial water membrane peeper patterned after the original design of Hesslein (1976). Prior to use at the study area, peeper compartments were filled with deionized water and the unit was allowed to degas at least eight hours under continuous bubbling with N_2.

After an equilibration period of 3-4 weeks, each peeper was retrieved and samples were obtained by piercing the dialysis membrane with a hypodermic needle, drawing the sample into a syringe, and filtering through a 0.22 μm membrane filter. Duplicate samples at each depth (2.5 cm) were composited in cases where the membrane was intact over both sample compartments. Care was taken to minimize exposure of the samples to the atmosphere.

Analytical Procedures

Sediment analysis. Sediment cores collected in the field were frozen upon arrival at the laboratory. Once thawed, the cores were sectioned at 2.5-cm intervals using a plastic knife. These sections were oven dried at 103°C, ground using a mortar and pestle, passed through a 2-mm sieve, and stored in polyethylene vials until analysis. All labware used in chemical analyses were acid-washed in 20% HNO_3, rinsed five times with distilled water, and rinsed five times with deionized water. All chemical reagents used were A.C.S. certified reagent grade.

Sediment pH was determined using 1:1 weight suspensions of ground sediment and water. Measurements were made after a 10 minute equilibration time.

A sequential extraction procedure (Fig. 1) was performed on ground sediment in order to determine the proportion of iron and manganese associated with diagenetically important sediment fractions. Sediment samples were extracted in 50-ml polycarbonate centrifuge tubes using appropriate reagents and centrifuged at 12,000 x *g* for 15 minutes. The supernatant was removed and acidified to $pH < 2$ with HNO_3. The residue was resuspended, centrifuged again, and the supernatant discarded. This procedure was performed after each successive extraction. Iron and manganese in the supernatant were determined by flame atomic absorption spectrophotometry (AAS) (Perkin-Elmer Model 703, Perkin-Elmer Corporation, Norwalk, CT).

Total organic carbon (TOC) was determined by dry combustion of 1:50 weight suspensions of ground sediment and water at a temperature of 800°C using a Dohrmann Model DC-80 automated total organic carbon analyzer (Xertex Corporation, Santa Clara, CA) and sludge/sediment sampler accessory. Total sulfur (TS) was determined using a LECO induction furnace (LECO Corporation, St. Joseph, MI).

Interstitial water analysis. Redox potential and pH were measured immediately after filtration. Each sample was then acidified to $pH < 2$ with HNO_3 for subsequent metal analyses. Redox potential was determined in the field with an epoxy-body redox platinum combination electrode. Measurements were noted after the readings stabilized (usually < 1 min). Values of pH were determined after the redox measurements with a glass-body pH combination microelectrode. Acidified samples were transported on ice to the laboratory and stored at 4°C until analysis for total iron and manganese using flame AAS.

Multivariate Statistical Analysis

Relationships between samples and between parameters were evaluated using cluster analysis and factor analysis, respectively. All data were standardized to mean zero and unit variance before analysis to remove the effects of mixed units. Ward's cluster analysis method was used to group the sediment samples according to the minimum variance criterion (Massart and Kaufman, 1983). Principle factor analysis was used to examine the sediment parameters for important interrelationships and to extract a small number of uncorrelated factors which explain the variances of the original parameters. To aid in the interpretation of these factors, the factors were rotated by the quartimax method so that the factor loadings approach 0 or ± 1 (Harman, 1967). Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC).

RESULTS

Description of Sediment Cores

All sediment cores collected exhibited marked differences in both color and texture with depth. In general, three zones could be identified: and upper zone (0.0-5.0 cm), a transition zone (2.5-7.5 cm), and a lower zone (greater than 7.5 cm). The upper zone was typically reddish brown to black and consisted of ferric hydroxide precipitates and partially decomposed organic matter, which abruptly changed into a transition zone consisting of light gray silt and clay, followed by a lower zone of light gray-tan silt and clay. Examination of concentration-depth profiles revealed that each zone had chemical characteristics unique to each zone.

Concentration-Depth Profiles

Concentration-depth profiles observed along both Seep S and Seep C revealed differences in both the horizontal and vertical distributions of all chemical variables studied. Sediment cores collected along both seeps showed variability between replicates as well as between stations. Descriptive statistics are shown in Table 1. In general, the surfical sediments were found to be enriched in iron and manganese, organic carbon, and sulfur, and the profiles show a trend of decreasing concentration with increasing depth (Fig. 2). Iron and manganese behaved differently, however, with respect to their partitioning between the five sedimentary fractions.

Iron. Total extracted iron, defined as the sum of all fractions, was considerably higher at Seep S (22,000-240,000 μg Fe/g dry sediment) relative to Seep C (13,000-25,000 μg Fe/g dry sediment) and varied with depth (Fig. 2a). Although total extracted iron varied considerably with depth, the partitioning of iron between the sedimentary fractions was relatively constant.

The exchangeable and carbonate fractions were negligible in comparison with other fractions. Reducible iron was most abundant at the sediment-water interface and decreased with depth. All metal fractions except carbonates were higher at Seep S relative to Seep C (Table 1).

The interstitial water concentration-depth profiles of total dissolved iron and manganese reflected the redox status of the sediment (Figs. 2c and 2d). The surficial sediment was oxidized with virtually no dissolved iron and manganese. Dissolved metal concentrations greatly increased at 2.5cm depth concomitant with a decrease in redox potential (Eh).

Oxidizable iron was a low percentage ($< 5\%$) of the total amount extracted and generally decreased with depth. Most of the iron in this fraction appeared to be associated with organic matter. The major iron fraction was residual and was primarily associated with clays, except near the sediment surface where it consisted primarily of crystalline iron oxides.

Manganese. Total extracted manganese was also higher at Seep S (180-48,000 μg Mn/g dry sediment) and varied considerably with depth (Fig. 2b). Only carbonate manganese was higher for Seep C sediments (Table 1). In contrast to iron, the partitioning of manganese between the sedimentary fractions was not constant with depth.

The exchangeable and carbonate fractions of manganese were more important than those of iron. Although exchangeable and carbonate manganese were most abundant near the sediment surface and decreased with depth, the percentage of exchangeable manganese approached 50% of the total manganese extracted.

At the surface, manganese oxides were very abundant and accounted for about 70% of the total manganese extracted. Reducible manganese was correlated with reducible iron and decreased until relatively constant values were observed at depths greater than 5.0 cm. Thus, changes in redox potential with depth (Fig. 2d) appeared to control the behaviour of iron and manganese.

As with iron, oxidizable manganese appeared to be associated with organic matter. Formation of MnS is promoted only under extreme reducing conditions at alkaline pH (Garrels and Christ, 1965). Residual manganese was associated with clays and became more important with increasing depth.

Sediment pH. Surficial sediment pH values were generally about one pH unit higher relative to sediment at depth (Fig. 2e). Seep S sediments were about one pH unit lower than those of Seep C (Table 1). The higher levels of carbonate monogynies in Seep C sediments are consistent with higher values of sediment pH. Although not measured at all stations, interstitial water pH was about one unit higher relative to sediment pH.

Organic carbon and sulfur. Relatively low levels of organic carbon and sulfur were observed in the sediments of both Seep S and Seep C. Total organic carbon ranged from about 2-6% (dry weight) at the surface to less than 1% at depth (Fig. 2f), while total sulfur ranged from less than 0.2% at the surface to less than 0.05% at depth (Fig. 2g). The low concentrations of oxidizable metals coupled with the relative lack of surface organic matter and low levels of sulfur suggest that the formation of metal sulfides is not significant. The high levels of total sulfur observed in the surficial sediments were correlated with high concentrations of reducible iron and may be due to the precipitation of ferric hydroxysulfates through the oxidative action of *Thiobacillus ferrooxidans* on ferrous iron.

Multivariate Statistical Analysis

Cluster analysis performed on the sediment data indicated that the samples can be grouped into four clusters based on their observed chemical characteristics. Cluster A consists of samples obtained from the lower depths of Seep S. Sediment samples from the upper depths of Seep S and the lower depths of Seep C are represented in Cluster B, illustrating the chemical similarity between the upper sediment zone of Seep S and the lower sediment zone of Seep C. Cluster C is indicative of the upper sediment zone of both seeps. Cluster D consists entirely of Seep S surface samples, all of which have are red-brown coloration characteristic of iron oxide precipitates.

The partitioning of iron for each cluster of samples is shown in Fig. 3a. Although total extracted iron varied considerably with depth, the partitioning of iron between the five sedimentary fractions was relatively constant. By far, the majority of sedimentary iron was residual (about 70% of total extracted iron). Of minor importance were the exchangeable and carbonate fractions, which constituted less than 2% of total extracted iron in most cases. About 20-25% of total extracted iron was reducible. Oxidizable iron accounted for about 5% of the total and was somewhat depth-dependent. Most of the iron in this fraction appeared to be associated with organic matter.

Manganese showed much more variability with depth than iron, both in the total amount extracted as well as metal partitioning (Fig. 3b). The majority of manganese at the surface (Cluster D) was reducible (about 70%). Exchangeable and residual manganese became more important with increasing depth (Clusters C,B,A). Oxidizable manganese accounted for about 8% of the total and appeared to be independent of depth.

Manganese associated with carbonates varied greatly with depth and was found to depend on pH. At Seep S, carbonate manganese was less than 10% of the total, while at Seep C it approached 50% of the total. This accounts for the higher percentage of carbonate manganese for samples in Clusters B and C.

Interrelationships between parameters were examined by correlation and factor analysis. The exchangeable, carbonate, reducible, oxidizable, and residual fractions of iron correlated well with those of manganese. Sediment pH correlated well with the metal carbonate fractions. Exchangeable metals were correlated with total organic carbon. Oxidizable metals were highly correlated with total organic carbon (Fe: $r = 0.812$; Mn: $r = 0.623$). The relationship between oxidizable metals and total sulfur, although statistically significant, was much less distinct (Fe: $r = 0.742$; Mn: $r = 0.516$).

Factor analysis was used to extract a small number of uncorrelated factors by identifying which parameters were simultaneously correlated. Four factors were extracted from the data (designated as outlined in Fig. 1) which explained 97% of the variance of the original thirteen parameters (Table 2): (1) an oxide-exchangeable metal-organic carbon factor; (2) a pH-carbonate metal factor; (3) an oxidizable Fe-sulfur-organic carbon factor; and (4) an exchangeable Mn-residual Mn-pH factor.

The least squares method was used to calculate factor scores for each sample (Harman, 1967). Mean factor scores were then calculated for each of the four clusters to determine the dominant relationships within each cluster. A large positive value of Factor 1 in the Cluster D indicates the importance of metal oxides and organic matter at the surface, and this factor became less important with increasing depth. The association of sediment pH with metal carbonates (Factor 2) was most important in Cluster C and least important in Cluster A, indicating the depth- dependent nature of this association. Factor 3 was highest in Cluster D and illustrates the association of oxidizable iron with organic matter at the surface and the correlation between organic carbon and sulfur. Factor 4, the least important factor, indicates an increase in exchangeable manganese by clay leaching at low pH value.

Organic Carbon-Sulfur Relationships

Carbon/Sulfur Ratios. The relationship between total organic carbon and total sulfur (Fig. 4a) was found to be statistically significant ($r = 0.854, p < 0.001$). The least squares regression line passes through the origin with a mean C/S ratio (slope^{-1}) of 22.5. C/S ratios on the order of 0.5-5 are typical of sediments rich in pyritic sulfur formed via bacterial sulfate reduction (Davison *et al.*, 1985). The relatively high C/S ration of 22.5 indicates that, on the average, little sulfate reduction has occurred in these sediments.

Relation to Oxidizable metals. The majority of oxidizable metals appeared to be specifically bound to organic matter and not associated with sulfides, based on the low levels of sulfur observed. A semi-logarithmic plot of organic carbon versus oxidizable metal concentration shows a curvilinear increase in oxidizable metals with increasing organic carbon (Fig. 4b), suggesting a decrease in the organic matter metal binding capacity with increasing depth (i.e., low organic carbon concentration).

DISCUSSION

Visual observation of all sediment cores collected revealed marked differences in both color and texture with depth which corresponded with changes in the distribution and forms of iron and manganese. Observed concentration-depth profiles may be explained in terms of a simple transport model as discussed by Davison (1982). Accordingly, accumulation of iron and manganese oxides at the sediment-water interface results from two processes: (a) the precipitation of oxidized metals from mine drainage surface water, and (b) an upward flux of reduced metals towards the surface along a concentration gradient. In this study the abundance of oxides found in the surficial sediments coupled with the location of the redox boundary and the shape of the dissolved metal concentration-depth profiles support this contention. Thus, aerobic conditions generally exist in the upper sediment zone (0.0-5.0 cm), although the location and intensity of the redox boundary are dependent upon the amount of decomposable organic matter and the depth of overlying water. Through sedimentation, oxidized metals at the sediment surface ultimately encounter reducing conditions, releasing soluble metals which then migrate towards the surface. There they may be oxidized and reprecipitated or incorporated into the sediment as reduced minerals, depending on the redox profile (Davison, 1982).

The upper sediment zone was found to have high levels of total sulfur in addition to an abundance of metal oxides. Although the major sulfur forms (sulfate, pyritic, and organic) were not distinguished, most of the sulfur in the upper sediment zone is probably in sulfate form. Precipitation of ferric hydroxysulfates and jarosites has been linked to the oxidative action of *Thiobacillus ferrooxidans* on ferrous iron (Wishlacz and Unz, 1986), and it seems likely that ferric hydroxysulfates and jarosites are present in the upper sediment zone.

The transition between aerobic and anaerobic conditions is assumed to be marked by the redox boundary. The presence of overlying water at the mine water seeps (usually less than 10 cm deep) and the aerobic decomposition of organic matter induce anoxia. The redox boundary is marked by a sharp Eh gradient and corresponds to the location of the transition zone between the upper and lower sediment zones. The establishment of reducing conditions is essential for the formation of reduced authigenic minerals such as pyrite (FeS_2) and rhodochrosite ($MnCO_3$).

Multivariate analysis indicated that an abundance of organic matter and metal oxides in the upper sediment zone (Clusters D, C) created adsorption sites for exchangeable iron and manganese (Table 2). Although cation exchange may be important in reducing acidity in AMD (Cronce *et al.*, 1980), the saturation of exchange sites with iron and aluminum leads to an increase in soil acidity (Clark, 1964). In this study exchangeable iron was only a minor fraction, supporting the limited role of cation exchange in iron retention (Wieder, 1988). The role of cation exchange in the retention of manganese remains unclear, however, Adsorption of manganese onto the surfaces of metal oxides and organic matter is appreciable in the aerobic zone, but under reducing conditions the transformation of exchangeable manganese to the soluble form releases manganese to the interstitial water (Patrick and Turner, 1968). This probably holds true for iron as well, although to a smaller extent since iron is much less mobile than manganese. The decrease of reducible iron and manganese with depth and the occurrence of dissolved iron and manganese in the interstitial water suggest the mobilization of these metals in reduced form.

Under anaerobic conditions in the lower sediment zone, oxidation of the remaining organic matter occurs by a succession of redox reactions involving nitrate, iron and manganese oxides, and sulfate. Typically, oxidation of organic matter by sulfate-reducing bacteria is the predominant decomposition reaction. Since a fraction of organic matter is refractory, some residual carbon is deposited in the sediment which has been found to be linearly related to the amount of sulfur present in sediments. This has led to the use of the organic carbon-sulfur ratio for interpreting the relationship between carbon and sulfur in sediments (Davison *et al.*, 1985).

The retention of iron and manganese within the sediments ultimately depends on the formation of reduced authigenic minerals (e.g. sulfides, phosphates, carbonates) in the anaerobic zone because oxidized metals in the aerobic zone are eventually solublized and released. A lack of decomposable organic matter at depth inhibits sulfate reduction, thus inhibiting the formation of authigenic metal sulfides. In typical marine sediments, sulfate reduction is limited by the amount and reactivity of sedimentary organic matter, while in freshwater sediments it is controlled by dissolved sulfate (Westrich and Berner, 1984). Thus, much more pyrite is formed in marine sediments. As a result, marine sediments exhibit much lower organic carbon-sulfur ratios (C/S) than freshwater sediments. Davison *et al.* (1985) reported marine C/S ratios in the range of 0.5-5 and freshwater C/S ratios in the range of 40-60. High ratios found in this study (C/S ˜ 22) indicate that, on the average, relatively little sulfate reduction has occurred in these sediments. Because of high sulfate inputs, sulfate reduction rates in AMD sediments would generally be limited by the amount and reactivity of sedimentary organic matter. This contention is supported by the plot of organic carbon versus sulfur (Fig. 4a). The zero intercept implies that all sedimentary organic matter was metabolized without the concomitant formation of sulfides; hence, all decomposable organic matter was degraded in the upper sediment zone where conditions were not anaerobic. Only a fraction of the more refractory organic carbon remaining in the lower sediment zone could be metabolized by sulfate-reducing bacteria, resulting in little sulfate reduction (Leventhal, 1983). Abundant surface organic matter and rapid sedimentation would likely lead to enhanced sulfate reduction and subsequent pyrite formation because a greater fraction of non-metabolized organic matter is preserved at depth. In organic-rich sediments, however, the flux of manganese to the overlying water by the dissolution of oxides may be more important than the formation of manganese carbonates and sulfides (El Ghobary and Latouche, 1986); thus, abundant organic matter may be both beneficial and detrimental to manganese retention.

CONCLUSIONS

The long-term potential for iron and manganese retention in wetlands receiving AMD appears to depend on the sediment redox profile. The majority of iron and manganese was retained in the aerobic zone in the form of metal oxides, but reduction and dissolution in the anaerobic zone released soluble metals to the interstitial water. Sulfide complexation was unimportant to metal removal in the anaerobic zone, presumably, due to insufficient available organic matter at depth.

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Table 1

Descriptive statistics for Seep S and Seep C sediment samples.

Parameter	Seep S			Seep C		
	Minimum	Maximum	Mean ± SE	Minimum	Maximum	Mean ± SE
Fe (ug/g)						
Exchangeable	6.8	480	41 ± 8.5	6.0	43	14 ± 1.3
Carbonate	8.6	160	30 ± 3.7	16	770	160 ± 31
Oxide	3,900	39,000	8,700 ± 730	3,200	7,900	4,700 ± 210
Organic/Sulfide	81	7,600	1,100 ± 178	560	3,500	1,400 ± 110
Residual	16,000	190,000	35,000 ± 3,800	8,800	19,000	14,000 ± 370
Mn (ug/g)						
Exchangeable	44	970	150 ± 20	14	320	110 ± 10
Carbonate	5.1	3,400	200 ± 65	21	4,200	710 ± 190
Oxide	47	37,000	2,200 ± 780	50	9,600	800 ± 310
Organic/Sulfide	9.3	8,200	230 ± 130	17	360	66 ± 12
Residual	60	1,800	180 ± 32	42	81	56 ± 1.6
Sediment pH	3.61	6.45	5.40 ± 0.08	5.19	7.13	6.36 ± 0.10
Total Organic Carbon (%)	0.19	5.58	1.47 ± 0.15	0.61	6.21	1.84 ± 0.21
Total Sulfur (%)	0.01	1.90	0.10 ± 0.03	0.03	0.28	0.08 ± 0.01

Table 2

Principal factor analysis of sediment samples.

Factor 1	Loading	Factor 2	Loading	Factor 3	Loading	Factor 4	Loading
Mn (3)	0.898	pH	0.819	Fe (4)	0.551	Mn (1)	0.383
Mn (4)	0.895	Fe (2)	0.806	TS	0.529	pH	-0.345
Fe (1)	0.835	Mn (2)	0.774	TOC	0.271	Mn (5)	0.317
Fe (3)	0.824						
Mn (1)	0.817						
TOC	0.724						
Fe (5)	0.644						
Variance Explained:							
52.7%		31.0%		7.8%		5.6%	
Cumulative:							
52.7%		83.7%		91.5%		97.1%	

(1)-Exchangeable Metals
(2)-Carbonate Metals
(3)-Reducible metals
(4)-Oxidizable Metals
(5)-Residual Metals

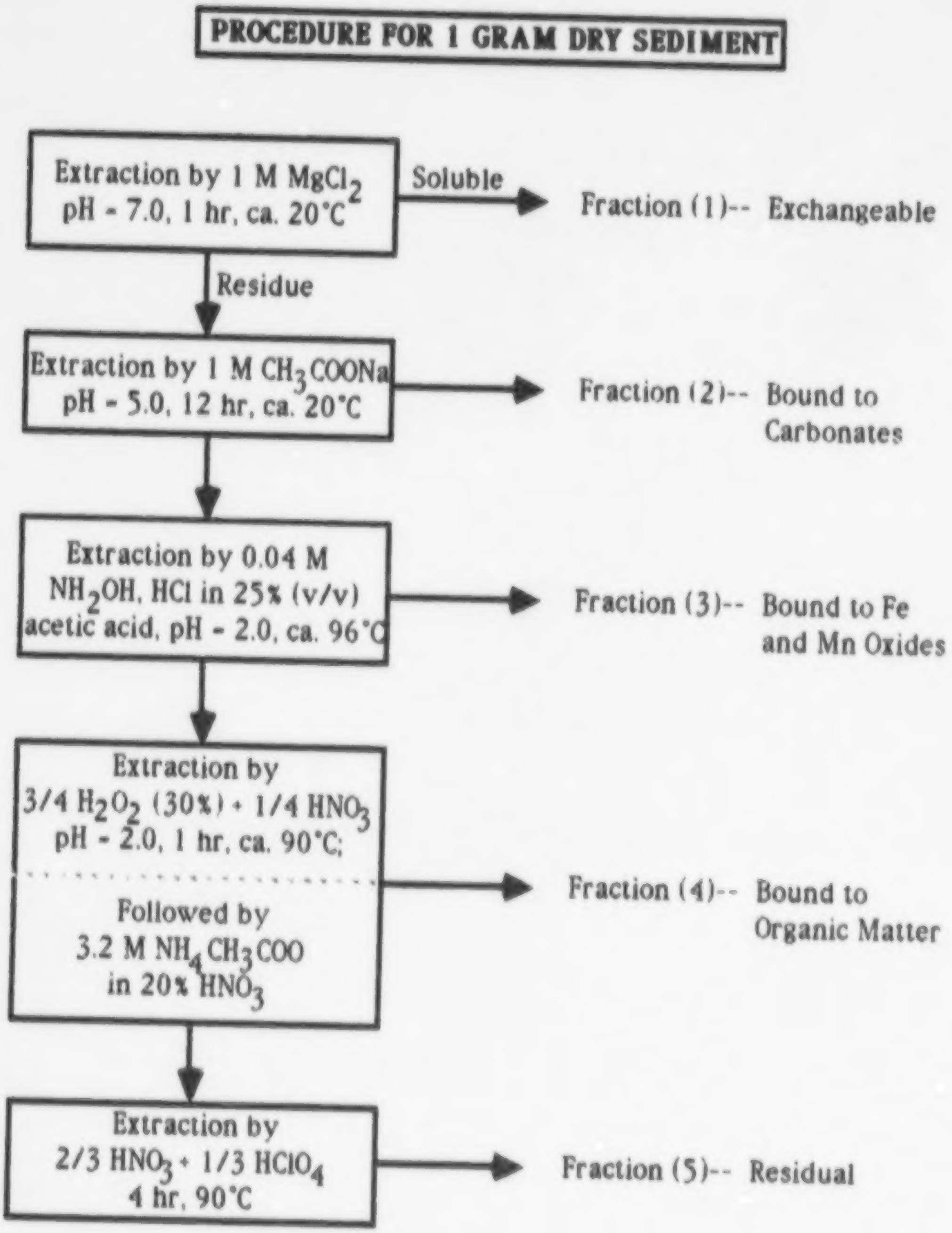

PROCEDURE FOR 1 GRAM DRY SEDIMENT
Extraction by 1 M $MgCl_2$ pH - 7.0, 1 hr, ca. 20°C
Soluble
Fraction (1)-- Exchangeable
Residue
Extraction by 1 M CH_3COONa pH - 5.0, 12 hr, ca. 20°C
Fraction (2)-- Bound to Carbonates
Extraction by 0.04 M NH_2OH, HCl in 25% (v/v) acetic acid, pH - 2.0, ca. 96°C
Fraction (3)-- Bound to Fe and Mn Oxides
Extraction by 3/4 H_2O_2 (30%) + 1/4 HNO_3 pH - 2.0, 1 hr, ca. 90°C;
Followed by 3.2 M NH_4CH_3COO in 20% HNO_3
Fraction (4)-- Bound to Organic Matter
Extraction by 2/3 HNO_3 + 1/3 $HClO_4$ 4 hr, 90°C
Fraction (5)-- Residual

Fig. 1 Sequential extraction procedure for determination of metal fractions in sediment (after Tessier *et al.*, 1979).

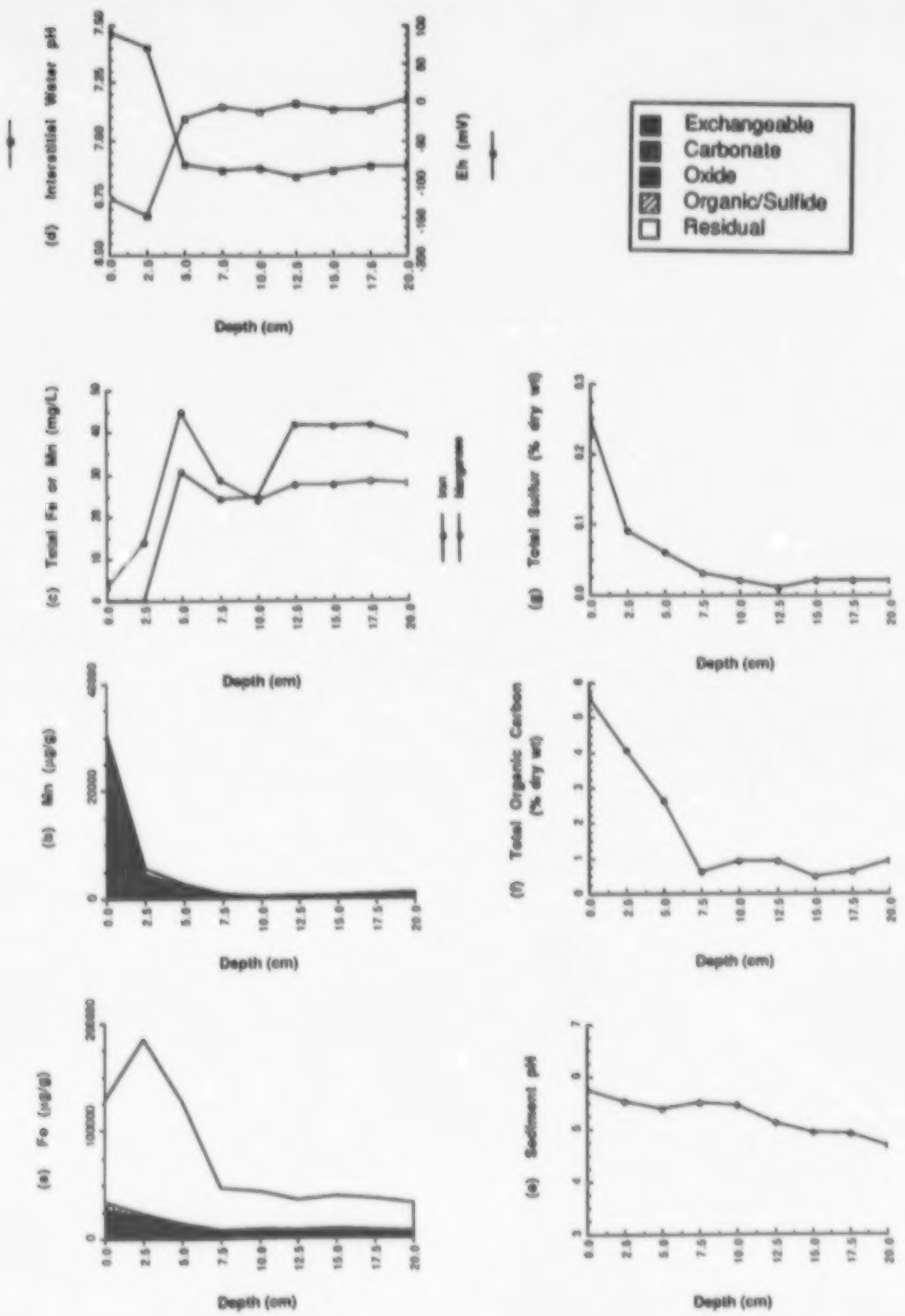
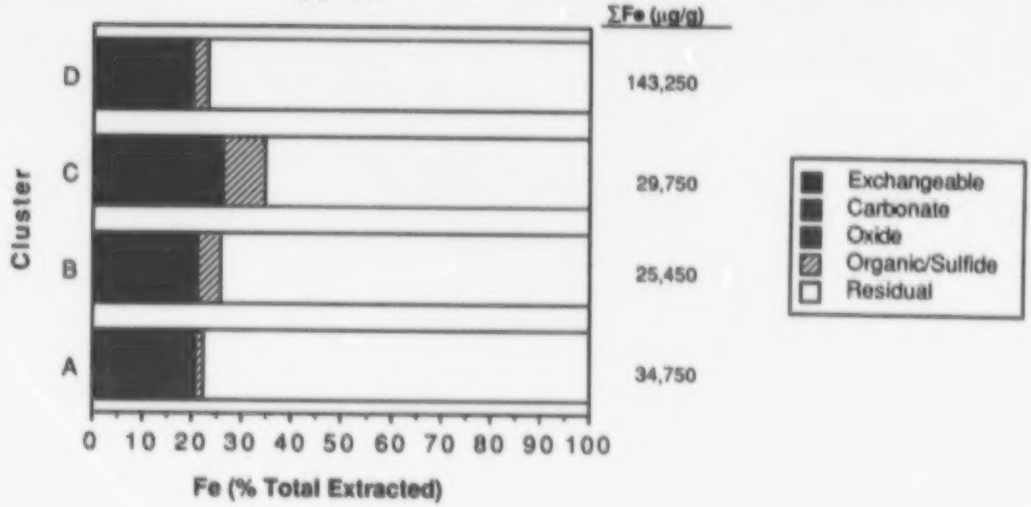


Fig. 2 Typical concentration-depth profiles of (a) sediment Fe; (b) sediment Mn; (c) interstitial water Fe and Mn; (d) interstitial water pH and Eh; (e) sediment pH; (f) total organic carbon; and (g) total sulfur.

(a) Iron



(b) Manganese

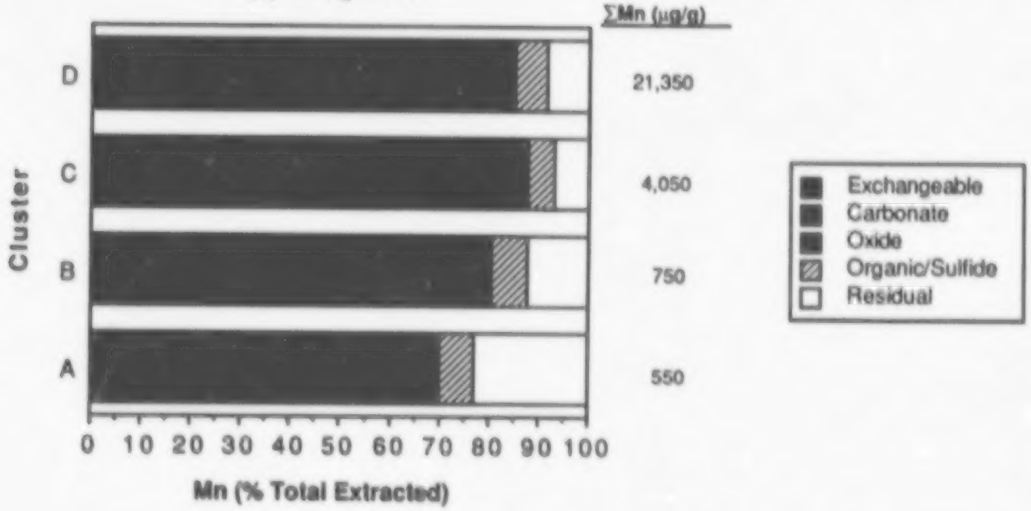


Fig. 3 Distribution of (a) total extracted Fe and (b) total extracted Mn among the five sedimentary fractions for each cluster.

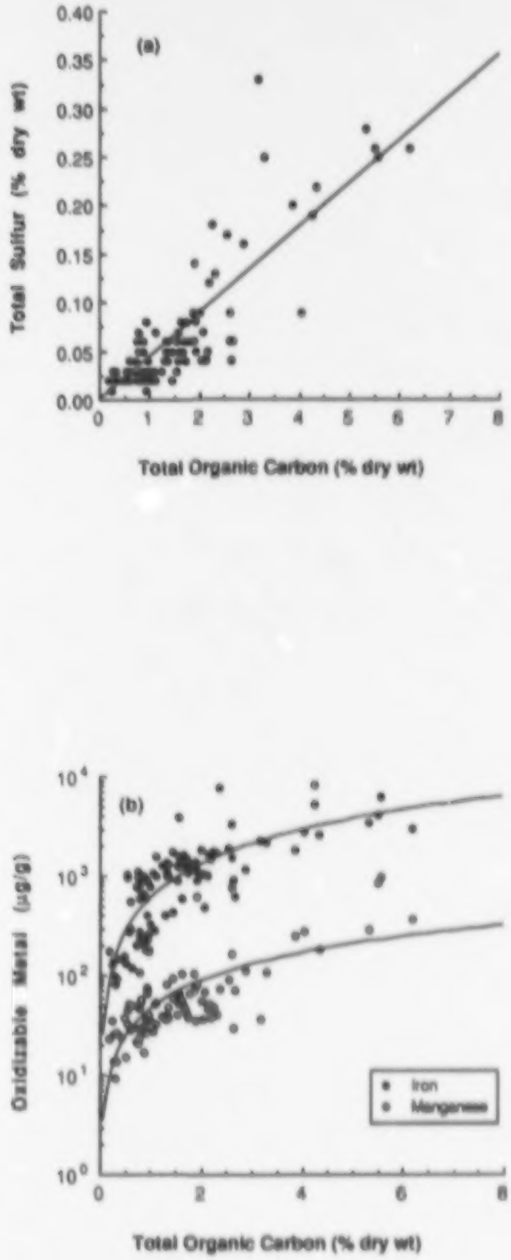


Fig. 4 Relationship between (a) total organic carbon and total sulfur and (b) oxidizable metals and total organic carbon.

Ecological Engineering Applied to Base Metal and Uranium Mining Wastes

M. Kalin

Boojum Research Limited, Toronto, Ontario

ABSTRACT

Ecological Engineering methods are decommissioning procedures for acid generating wastes from base metal and coal operations. They entail the enhancement of natural processes which occur in wetlands and in sediments. Those are microbial, sulphate and iron reduction and the relegation of metals to the sediment through biological ad/absorption and precipitation of iron hydroxides. These methods have been implemented for the abandonment of acid generating tailings from a copper/zinc concentrator in northern Ontario, the AMD seepage from a coal lifting and banking centre and for the seepage from an abandoned coal dump, both in Cape Breton, Nova Scotia, Canada. This paper presents the significant factors which govern the success of the application of ecological engineering methods. Those are contaminant release rates, growth and ad/absorption rates, sulphate and iron reduction rates, as well as the bio/geochemistry of the metal to be removed from the water column.

LE GÉNIE ÉCOLOGIQUE: SON APPLICATION AUX MÉTAUX COMMUNS ET AUX RÉSIDUS D'URANIUM

M. Kalin

Boojum Research Ltée
Toronto (Ontario) Canada

RÉSUMÉ

Les méthodes de génie écologique visent à neutraliser les résidus d'extraction du charbon et les métaux alcalins qui peuvent former de l'acide. Elles tentent à reproduire les phénomènes qui ont cours dans les marais et dans les sédiments, soit la réduction microbienne du fer et du sulfate et l'emprisonnement des métaux dans les sédiments par des procédés d'adsorption et d'absorption biologique et de précipitation d'hydroxyde de fer. Ces méthodes ont été utilisées dans le cas de résidus provenant d'un concentrateur cuivre/zinc dans le nord de l'Ontario, pour les liquides de suintement acides d'un poste d'amenée et d'entassement du charbon et d'une décharge de charbon abandonnée, tous deux au Cap-Breton en Nouvelle-Écosse, au Canada. Cet article présente les facteurs importants qui déterminent le succès des méthodes de génie écologique, soit la vitesse d'écoulement du contaminant, les taux de croissance, d'adsorption et d'absorption, les vitesses de réduction du fer et du sulfate ainsi que les caractères biogéochimiques du métal à extraire de la colonne d'eau.

INTRODUCTION

Ecological Engineering methods involve the design of ecosystems for the benefit of both mankind and the natural environment (Mitsch and Jorgensen, 1989). Similar to other forms of engineering and technology, Ecological Engineering uses basic principles of science and engineering to match disturbed environments with new ecosystems, but then allows these ecosystems to self organize into maintenance-free systems (Odum, 1983).

That long-term solutions to environmental problems generated by base metal and coal mining waste will emerge with development of Ecological Engineering methods has been proposed in concept by Kalin (1986). The aim of Ecological Engineering methods developed to date for mine waste management is to facilitate the establishment of self-sustaining ecosystems which yield environmental acceptable conditions upon closeout of mine/mill waste sites. The first phase of investigations of base metal wastes led to the definition of; a biological polishing system utilizing several attached algal groups for acidic or circumneutral waste water; a biological filtration system; vegetation covers which induce chemical reducing conditions (Kalin and R.O van Everdingen, 1988). This paper summarizes briefly the progress made to date in the development and application of Ecological Engineering methods for uranium, base metal and coal mining wastes.

METHODS OF ECOLOGICAL ENGINEERING

Natural contaminant removal processes comprising Ecological Engineering employ bio-geochemical and biological components, in addition to physical processes, all common to natural ecosystems. In short, these Ecologically Engineered systems should be able to sustain themselves indefinitely. In Schematic 1, those processes principally responsible for natural contaminant removal are depicted. As the ecosystems are driven by solar energy, biochemical and biological processes of the system are not dependent on technological energy sources.

As specific conditions are required for the capture and removal for a contaminant according to its type and chemical state, the design of an ecologically engineered system must provide a choice of conditions which, through ecosystems' self-designing capacity, will generate an ecosystem matching the chemical environment. The processes of natural removal of zinc and Ra^{226} provide good examples of this specificity.

Zinc removal from the water column is driven by adsorption on, and co-precipitation with, manganese oxides and amorphous iron oxides, processes depicted in the centre of Schematic 1. Furthermore, removal of zinc may proceed by adsorption onto organic matter under acidic conditions, although the affinity of organic substrates may be affected by pH, Eh, and the ambient mineralogy and organic acid chemistry. These processes are depicted on the right hand side of the Schematic 1.

The removal of dissolved Ra^{226}, in contrast to zinc, is governed mainly by chemical and biochemical factors rather than involving processes such as adsorption onto organic substrates. Co-crystallization is a primary process, where Ra^{226} substitutes for barium in barium sulphate, for calcium in calcium carbonate, or for iron and manganese in their oxides or hydroxides. As well, Ra^{226} can co-precipitate with iron-arsenic compounds. Rs^{226} removal may also proceed by adsorption onto iron and manganese oxides, clay minerals and organic matter, processes depicted on the left and right-hand sides of the schematic.

ECOLOGICAL ENGINEERING: SITE SPECIFIC APPLICATIONS

To date, an Ecological Engineering system has been developed for the removal of dissolved zinc from acid mine drainage waters, as well as for the removal of Ra^{226} and uranium from alkaline waste waters.

Base metal tailings and acid mine drainage are harsh habitats, where relatively few biota are tolerant of, let alone indigenous to, the mining waste material. Habitat is characteristically nutrient poor, with extremes in chemical (acidic or alkaline) and physical (exposure and drought) conditions (Kalin, 1983, 1984). Biota, such as *Typha* spp., typically populate only isolated areas on these waste sites. Extensive colonization of waste sites and establishment requires plant populations with physiological mechanisms allowing germination, establishment and finally growth in these harsh environments (Kalin and Caza, 1982).

The ongoing work addresses the development of methods to create those specific conditions stimulating spread of these indigenous species beyond the isolated areas on the waste site. Only with a thorough understanding of the establishment and growth factors can this be accomplished.

Ecological Engineering of these waste areas will reduce the rate of acid generation and subsequent contaminant release. The development of appropriate biological covers which reduce infiltration of water and increase evapotranspiration (Kalin, 1987), as well as diversion of surface water away from waste material and utilization of available covers such as mine slimes will all reduce rates of contaminant release.

Water quality improvements can be expected through the integration of precipitation processes in interceptor ditches and polishing ponds. Biological polishing processes facilitate the adsorption of metals onto algal biomass, which is subsequently relegated to the sediment (Kalin, 1988). Finally, systems are being tested where water is passed through sulphate-reducing environments where microbial conversion of sulphate to hydrogen sulphide results in metal precipitation (Cairns et al.,1988; Kalin *et al.*, 1989).

CONCLUSIONS

Progress in this area of technology, presently reported for acid mine drainage in base metal and coal mining, and alkaline effluents generated by uranium mining, indicates that man's assistance is required primarily during the critical period of biota's establishment; it is to this end that all research to date has been directed. This establishment phase's eventual success is dependent upon the effectiveness of the initial work performed. Presently, the effectiveness of Ecologically Engineered systems established on several waste sites is being evaluated and ascertained.

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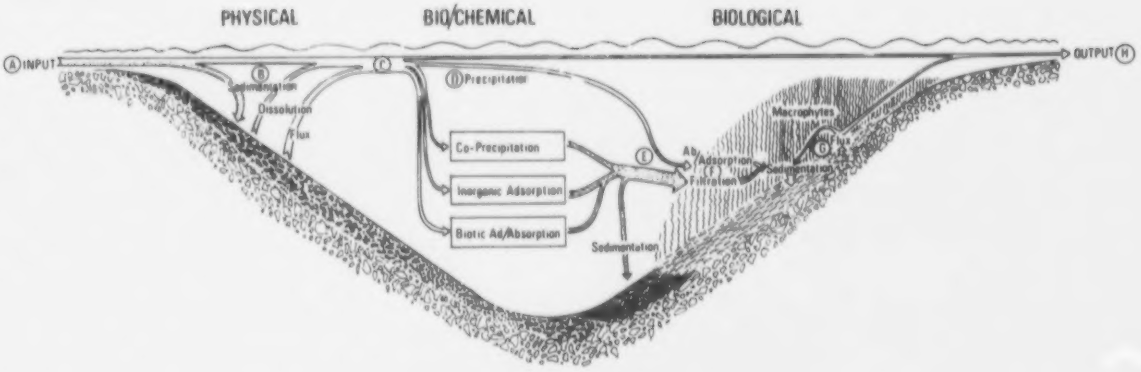
PHYSICAL
BIO/CHEMICAL
BIOLOGICAL
Ⓐ INPUT
OUTPUT Ⓗ
Ⓑ
Sedimentation
Dissolution
Flux
Ⓒ
Ⓓ Precipitation
Co-Precipitation
Inorganic Adsorption
Biotic Ad/Absorption
Ⓔ
Sedimentation
Ab/Adsorption
Ⓕ
Filtration
Macrophytes
Ⓖ
Flux
Sedimentation

Schematic 1: Ecological Engineering: The processes which apply to contaminant removal.

ACCUMULATION AND PRECIPITATION OF Cd AND Zn IONS BY ALCALIGENES EUTROPHUS STRAINS

L. Diels

Laboratory of Genetics and Biotechnology, S.C.K.-C.E.N., B-2400 Mol, Belgium

ABSTRACT

Alcaligenes eutrophus var. metallotolerans is a typical colonizer of soils with high levels of heavy metals. Type strain *A. eutrophus* CH34 carries large plasmids responsible for resistance against Cd^{++}, Co^{++}, Zn^{++}, Hg^{++}, Tl^{+}, Cu^{++}, Pb^{++}, Ni^{++} and $Cr0_4^{-}$. Resistance mechanisms of Cd, Zn and Ni during exponential growth phase and at low metal concentration seem to work by a lowered metal uptake due to ATP driven efflux systems. On the other hand, at least for Cd and Zn, an accumulation of up to 500 μg metal/mg Dry Cell Weight in the stationary phase was observed. In this growth phase, higher amounts of extracellular polysaccharides were observed at Cd concentrations higher than 0.5 mM. Accumulation increases with high concentrations of carbon sources (lactate, gluconate) and phosphate. Otherwise, due to the slow alkalinization of the culture medium, $CdCO_3$ and $Cd(OH)_2$ were formed as could be identified by IR spectroscopy. Bacteria are supposed to serve as a primary inoculum for crystallization of Cd salts. In this way up to 2 mM Cd or 10 mM Zn could be removed from a contaminated solution. Also soil leachates with low heavy metal concentrations could be cleaned.

ACCUMULATION ET PRÉCIPITATION DES IONS Cd ET Zn PAR DES SOUCHES D'*ALCALIGENES EUTROPHUS*

L. Diels

Laboratoire de Génétique et de Biotechnologie
Ministère de l'Environnement et de l'Énergie
S.C.K.- C.E.N., Mol, Belgique

RÉSUMÉ

Alcaligenes eutrophus var. metallotolerans CH34 a été isolée à partir d'un bassin de décantation d'une usine de zinc. La souche possède deux grands plasmides qui codent pour la résistance au Cd^{++}, Co^{++}, Zn^{++}, Hg^{++}, Tl^{+}, Cu^{++}, Pb^{++}, Ni^{++} et CrO_4^- . La résistance au Cd, Sn, Cr et Ni pendant la phase de croissance exponentielle et à des concentration de métaux faibles semble être due à une diminution de l'absorption de métaux en raison d'un système d'écoulement dépendant de l'ATP. Toutefois, pendant la phase stationnaire, on a pu observer des concentrations de métaux allant jusqu'à 500g/mg (poids cellulaire sec), du moins pour le Cd et le Zn. Pendant la phase de croissance, on observe des concentrations plus élevées de polysaccharides lorsque la concentration de Cd est supérieure à 0,5 µM. L'accumulation augmente lorsque les concentrations de C et de P sont élevées. Dans le cas contraire, en raison de l'alcalinisation très lente du milieu de culture, il y a formation de $CdCO_3$ et de $Cd(OH)_2$, comme le prouve la spectroscopie à infrarouges. Les souches CH34 pourraient probablement servir d'inoculum principal pour la cristallisation des sels de Cd. De cette façon, on pourrait extraire jusqu'à 2 µM de Cd ou 10 µM de Zn d'une solution contaminée. De plus, les produits de lixiviation du sol dont les concentrations en métaux sont faibles pourraient être nettoyés. Le transfert de gènes de résistance aux métaux lourds à d'autre souches est une nouvelle façon de surmonter les problèmes d'inhibition causés par les métaux lourds dans d'autres souches bactériennes utilisées dans l'industrie.

INTRODUCTION

During the last years, bacteria resistant to a variety of heavy metals were isolated and identified (for review, see Silver, 1988). The mechanisms for these resistances are often controlled by plasmid borne genes or by transposons. A remarkable example of these resistant bacteria is *Alcaligenes eutrophus* var. *metallotolerans*. The representative strain CH34 was isolated in sediments from a decantation basin of a zinc factory (Mergeay *et al.*, 1978). Strain CH34 bears two large plasmids controlling resistance against Cd^{++}, Co^{++}, Zn^{++}, Hg^{++}, Tl^{+}, Cu^{++}, Pb^{++} (pMOL30, 240 kb) and Co^{++}, Zn^{++}, Ni^{++}, Hg^{++}, CrO_4^{-}, Tl^{+} (pMOL28, 165 kb). On pMOL28 nickel, cobalt and zinc genes are probably on the same cluster. One DNA fragment (in pMOL30) of about 9 kb (Nies *et al.*, 1987) is responsible for the resistance against cadmium, cobalt and zinc (czc^{+} fragment). Both plasmids contain a mercury transposon: Tn4378 and Tn4380 (Diels *et al.*, 1985, 1989a). Different heavy metal resistance genes could be cloned namely the Cd-Co-Zn genes (czc^{+}) (Nies *et al.*, 1987), the Hg genes (Diels *et al.*, 1985) and the Co, Ni, Cr genes (Nies *et al.*, 1989c; Siddiqui *et al.*, 1989). The czc^{+} and Mer-Tha genes were used as probes for hybridization with total DNA from strains isolated from different mining and industrial sites in Belgium and Zaire (Diels *et al.*, 1988a,b). From these different sites, strains hybridizing with our probes could be isolated (Diels *et al.*, submitted). The showed an impressive convergence of phenotypic traits with strain CH34 and especially for the presence of megaplasmids banding in the same region, the multiple resistance to heavy metals, and a typical carbon source pattern. From data on nickel resistance and electrophoretic pattern of proteins, two subgroups can be indicated : a Ni resistant and a Ni sensitive group. Bacteria from this Ni sensitive group carry a czc+ plasmid able to transfer at high frequencies (up to 10^{-3} per donor). Most of the Ni resistant strains give a temperature (37°C) induced mutagenesis (Sadouk, 1989). As could be shown by Nies *et al.*, (1989a,b) resistance to chromate is inducible and based on decreased net accumulation of the metal anion and resistance to zinc, cadmium, cobalt and nickel are resulting from inducible, energy dependent cation efflux systems. In the present communication, we show that in some physiological circumstances *A. eutrophus* can also accumulate heavy metals, which could lead to some applications in metal sequestration.

MATERIALS AND METHODS

Bacterial strains

Used strains of *A. eutrophus* are reported in Table 1.

Media

The cells were grown on a Tris mineral salts medium (Schlegel et., 1961) provided with gluconate (0.2%, wt/ml) or lactate (0.8%, wt/ml) as carbon sources.

Cd uptake

A sterile Cd solution was added aseptically to the medium. To each culture 0.5 μCi ^{109}Cd was added. Bacterial Cd sequestration during stationary phase was measured by the method of Mowl and Gadd (1984). A silicone oil : donylphtalate (2.8:2.2) layer was used to allow the immediate separation of cells from culture medium. The cells were centrifuged into a 30% perchloric acid solution and ^{109}Cd in the pellet was measured in a scintillation counter.

Cd removal and pH control

For Cd removal studies, shaken cultures of 250 ml were centrifuged and Cd was measured in the supernatant by plasma emission spectroscopy. Cultures with pH control were grown in a SETRIC Genie Industriel (Set 2) chemostat and when indicated pH was maintained at 7 by automatic addition of lactic acid.

Chemicals

Analytical grade $CdCl_2 \cdot 2\ 1/2\ H_20$ (UCB, Belgium) and $ZnSO_4 \cdot 7H_20$ (S.T. Baker, Holland) were prepared as 1.0 M stock solutions. Radioactive $^{109}CdCl_2$ with a specific activity of 140 mCi/μmol was obtained from Amersham (U.K.). Silicone oil DC.550 and dionylphtalate were obtained from Fluka (Switzerland). All other chemicals were obtained from E.Merck A.G. (Darmstadt, FRG). Soil leachate was prepared by shaking soil with a solution (0.8% lactic acid) of pH 2 at a ratio of 1 to 4 for soil to water during 15 minutes.

The soil was removed by centrifugation and the supernatant used as culture medium adjusting the pH to 6 with NH_4OH and adding 0.9 mM glycerophosphate.

RESULTS

Cd^{++} accumulation during stationary phase

When Cd^{++} uptake was measured (with gluconate as carbon source) at the beginning of the stationary growth phase (Figure 1), it was observed that at concentrations permissive for the Cad$^-$ strains, these accumulate more Cd^{++} that the Cad$^+$ strains (thus provided with pMOL30) (Nies *et al.*, 1989a,b) ; but, at high concentrations, the resistant strains may take up very substantial amounts of Cd^{++} (130 μg Cd/mgr Dry Cell Weight).

If higher concentrations of lactate (0.8%) were used, Cd^{++} sequestration from the medium was accompanied by progressive precipitation of a light milky material fixed on the glass vessels. This material was harvested, analyzed by infra-red spectroscopy, and was found to contain a mixture of $CdCO_3$ and Cd hydroxydes (P. Nagels, pers. comm.).

Table 2 shows that under these conditions (Tris minimal medium with 0.8% lactate) cell bound cadmium can rise up to 50% of dry weight when more than 0.4 mM of phosphate is used. The influence of the phosphate source also generated mineral precipitation of cadmium salts ($CdHPO_4$) using bacteria as a support (Macaskie *et al.*, 1987).

Figure 2 shows the removal of Cd from a shaken culture by CH34 in function of the growth curve. Lactate concentration was 0.8% (wt/ml) and 0.9 mM of phosphate were used. Cd^{++} was efficiently removed from the medium especially in late log phase and stationary phase. At the end of the culture, the medium contained 40 μM of Cd^{++}, 50 times less than the initial concentration (2 mM).

Other results show an increase of outer membrane proteins and extracellular polysaccharides by a factor 2.5 (Diels *et al.*, 1989c) in function of Cd concentrations increasing up to 2 mM.

pH increase is responsible for the accumulation of Cd^{++} observed

The progressive increase of the pH seems to be an essential feature in the observed accumulation. Therefore, bacterial growth and Cd^{++} removal were monitored in cultures with pH permanently maintained at 7. In such conditions, Cd removal from the culture was limited and the amount of cell bound Cd^{++} was very markedly decreased. Also no increase of extracellular polysaccharides or outer cell membrane proteins could be observed (Diels, 1989b).

Zn^{++} and Cd^{++} removal from contaminated solutions

Data from Table 3 show that the removal of Cd and Zn from solutions containing respectively 2 mM Cd or 10 mM Zn is effective only with lactate as a carbon source and not with citrate.

Table 4 shows the removal of heavy metals from a leachate of contaminated soil. The pH of the leachate was adjusted to 6 and 1 mM of glycerophosphate was added. Then the solution was shaken with CH34 during 2 days of heavy metal content was measured in the supernatant. Table 4 shows that at least the Cd and Zn concentration are below 0.5 ppm after bacterial treatment.

DISCUSSION AND CONCLUSIONS

At increased concentrations of Cd^{++}, accumulation of Cd^{++} in bacterial cells is observed during the late log phase and the stationary phase : this accumulation is correlated with the concentration of the carbon source (lactate or gluconate), with the progressive alkalinization of the medium, with the phosphate concentration and appears to be associated exclusively with the membrane. Precipitation of $CdCO_3$, $CdHPO_4$ etc. seems to happen on the membrane.

A possible interpretation would be that cadmium speciation would change due to the progressive pH increase and that the metal is trapped on the cell envelopes by precipitation using a membrane component as a support. Probably there are high pH gradients building up on the cell wall before reaching the solution. This is supported by the observation that Cd removal was markedly decreased in cultures with a pH permanently maintained at 7. Hambuckers-Berhin and Remacle (personal communication) showed high amount of Cd binding to the peptidoglycan layer, thus in the periplasmic space. This trapping would occur either at the uptake of Cd^{++} or at the efflux or more likely during both processes and suggests a kind of biomineralization as reviewed by Mann (1988).

As shown in Table 3 and 4, *A. eutrophus* CH34 can remove high concentrations as well as low concentrations of Cd and Zn from a minimal medium with some added nitrogen, phosphate, and lactate as carbon source, but not citrate (due to its high metal complexing capacity).

More than 2 mM of Cd or 10 mM of Zn can be removed by the bacterial treatment. When lactate is used as carbon source a very good accumulation can be obtained. These Cd and Zn concentrations are comparable to those found in environments such as mine refuses, sediments from non-ferrous industries and areas decertified by aerial deposits of zinc.

Sometimes bacteria are inhibited in their metabolism by the presence of toxic concentrations of heavy metals. Therefore it would be useful to overcome these inhibition problems by transferring heavy metal resistance genes to strains of industrial interest. Therefore we constructed new strains by transfer of the heavy metal resistance plasmids pMOL30 or pMOL28 to the PCB degrading strain A5 (containing pSS50) or vice versa. In that way Cd, Zn or Ni resistant PCB degrading strains were created. This combination of heavy metal resistance and xenobiotic catabolism can be of particular interest for waste treatment processes where mostly different contaminants are encountered together. Transfer of heavy metal resistance genes cloned on broad host range vectors to leaching bacteria (chemolithotroph or heterotrophs) can probably be of interest in overcoming inhibition caused by some dissolved heavy metals during the leaching process.

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Table 1

Bacterial strains

Strain	Plasmids	Relevant Plasmid Markers
CH34 (wild type)	pMOL30, pMOL28	Cad$^+$, Zinc$^+$, Nic$^+$
AE128	pMOL30	Cad$^+$, Zin$^+$
AE126	pMOL28	Nic$^+$
AE104	—	—

Table 2

Cd^{++} binding as a function of phosphate concentration.

Phosphate concentration (mM)	Remaining Cd ppm	Bound Cd %	Final pH
0.146	NM	4.8	—
0.225	6.9	9.5	7.72
0.450	2.0	44.2	7.84
0.311	3.2	25.9	7.83
0.625	1.1	39.8	7.86
0.933	1.0	49.7	7.87

Cd^{++} binding was measured in a culture of strain CH34 provided with lactate (0.8%) and an initial Cd^{++} concentration of 2 mM at different phosphate concentrations. The remaining Cd was the concentration of Cd found in the supernatant of the culture after centrifugation.

Bound Cd was measured on the cells expressed in percentage of Cd per dry well weight.

Table 3

Removal of Cd and Zn by CH34

Metal	Condition	Metal concentration (ppm)			
		origin	4 days	5 days	7 days
Cd	lactate pH 5.5	230.0	5.9	4.3	5.9
Zn	lactate pH 5.5	615.0	421.0	308.6	87.0
Cd	lactate pH 7	226.4	4.7	3.3	2.3
Zn	lactate pH 7	612.0	9.1	7.8	5.1
Cd	citrate pH 5.5	228.0	212.4	212.0	217.2
Zn	citrate pH 5.5	636.5	669.0	602.5	660.0
Cd	citrate pH 7	227.8	223.4	223.0	230.6
Zn	citrate pH 7	631.5	685.0	686.0	679.5

Solutions contaminated with 2 mM or Cd or 10 mM Zn were shaken in a minimal medium with lactate or citrate at pH 5.5 or 7. Metal measurements were done in the supernatant after centrifugation of the bacteria.

Table 4

Heavy metal removal of a contaminated soil leachate

	Metal concentration		
time	Cd	Cu	Zn
0 days	3.5	2.8	59.4
1 day	2.2	1.6	13.2
2 days	0.1	1.2	0.3

Table 5

pKa values of acid and basic functions of CH34

	pKa values	
culture conditions	acid function	basic function
wihout Cd	–	10.7
with 2 mM Cd	5.2 2.7	9.5

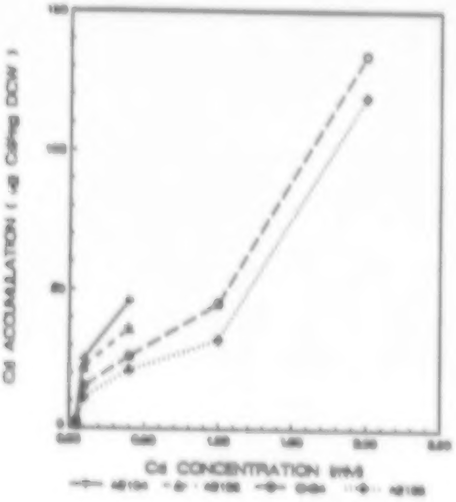

Fig. 1 Accumulation of cadmium in function of the initial cadmium concentration.

A. eutrophus strains were grown for 50 hours in a mineral salt medium provided with gluconate (0.2% as a carbon source. Cad⁺ (CH34 and AE128) and Cad⁻ strains (AE126 and AE104) are shown.

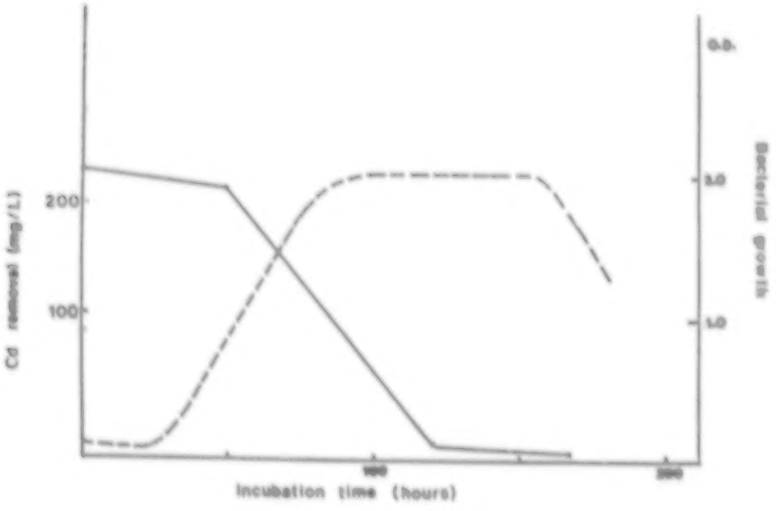

Fig. 2 Cd removal from a culture medium in function of growth of *A. eutrophus* CH34.

A culture medium (glycerophosphate as P source and lactate as C source) with 2 Mm Cd was inoculated with CH34.

At different times culture samples were taken, optical density (–) measured and centrifuged. The Cd remaining in the supernatant (–) was measured by plasma emission spectroscopy.

BASIC MICROBIOLOGY

PHYLOGENY OF ACIDOPHILIC BACTERIA ASSOCIATED WITH MINERAL LEACHING

David J. Lane, Gen-Trak Systems, Framingham, MA 01701

and

Arthur P. Harrison, Jr., Division of Biological Sciences, University of Missouri, Columbia, MO 65211*

Abstract

Phylogeny of Thiobacillus spp. was determined by means of base sequences in 16S rRNA. These bacteria have been placed in relationship to other sulfur- and iron-oxidizers and also heterotrophic bacteria. The several DNA Homology Groups of *T. ferrooxidans* differ from each other (in "evolutionary distances") to about the same magnitude as they differ from T. thiooxidans. These two species, with un-named species DSM 612, form a cluster of common descent. "*Leptospirillum ferrooxidans*", vibrioid iron-oxidizers, may represent a new phylum. The moderate thermophiles, strains Alv, BCl, and TH3, show affinity with Gram positive bacteria. Sulfur- and iron-oxidizing species also occur in the Archaebacteria. The genes for chemolithotrophy have arisen several times, the relevant species representing evolutionary convergence; or, on the contrary, these genes are ancient, and have been retained in otherwise divergent species. Perhaps segments of these genes serve other functions in heterotrophic bacteria and for organotrophic growth by facultative species of sulfur- and iron-oxidizing bacteria.

PHYLOGÉNIE DES BACTÉRIES ACIDOPHILES ASSOCIÉES À LA LIXIVIATION DES MINERAIS

D. J. Lane Gen-Trak Systems, Framingham, Maine É.-U.

et

A.P. Harrison Jr., Division des Sciences Biologiques, Université du Missouri, Columbia, Missouri, É.-U.*

RÉSUMÉ

La phylogénie d'espèces bactériennes chez *Thiobacillus* a été établie à l'aide des séquences de base de l'unité 16S de l'ARNr. Ces bactéries ont été classifiées en fonction de d'autres bactéries capables d'oxyder le soufre et le fer ainsi qu'avec des bactéries hétérotrophes. Plusieurs groupes d'ADN homologues chez *Thiobacillus ferrooxidans* diffèrent autant l'un de l'autre en termes de position sur l'échelle de l'évolution qu'ils diffèrent de *Thiobacillus thiooxidans*. Ces deux espèces bactériennes, ainsi que l'espèce DSM 612 (sans nom), partagent la même origine. *Leptospirillum ferrooxidans*, vibrion oxydant le fer, peut constituer un nouveau phylum. Les bactéries modérément thermophiles, des souches Alv, BC et TH3, montrent un certaine affinité avec les bactéries Gram positif. Les espèces oxydant le soufre et le fer existent aussi chez les archéobactéries. Les gènes responsables de la chimiolithotrophie ont été décelés à plusieurs reprises, les espèces en question étant convergentes du point de vue de l'évolution. Ces mêmes gènes peuvent aussi être anciens et avoir été retenus par des espèces autrement divergentes. Dans le cas des bactéries hétérotrophes, il se peut que des segments de ces gènes aient d'autres fonctions ou qu'ils soient associés à la croissance organotrophe des espèces bactériennes facultatives oxydant le soufre et le fer.

INTRODUCTION

Bacterial lithotrophy has been viewed as something disparate, so striking and exotic as to warrant a major role in structuring phylogenetic systems. For example, Orla-Jensen (1909) place those chemoautotrophs which can use atmospheric hydrogen as source of energy in his genus *Hydrogenomonas*, and Beijerinck (1904) assigned autotrophs which use sulfur as energy source in the genus *Thiobacillus*.

Hydrogenomonas was short-lived. Because its species were facultative, that is, could also use organic substrates for energy, they were dispersed to other genera, most to *Pseudomonas*. (Compare the 8th edition of *Bergey's Manual of Determinative Bacteriology*, 1974, p. 218, with the 6th edition, 1948, p. 76.) *Pseudonomas* as a taxonomic "catch-all". This view became fact when Palleroni *et al.* (1973) used nucleic acid homology to establish phylogenetic relationships within *Pseudomonas*, and demonstrated that some species are not much more closely related to one another than to *Escherichia coli*.

Thiobacillus fared better. When, here, facultative species were encountered, species capable of using as energy either sulfur or organic substrates, they were held within *Thiobacillus*. It was deemed more convenient to emphasize common sulfur catabolism than to submerge these facultative species within *Pseudomonas*, already a too broadly-defined genus. London & Rittenberg (1967) called attention to the unavoidable subjectivity in making this choice.

A *Thiobacillus* capable of oxidizing Fe^{++} as source of energy was described (Temple & Colmer, 1951), and was aptly named *Thiobacillus ferrooxidans*. Thus, two lithotrophic traits are displayed by a single species. Is there a reason why one trait should be, phylogenetically, subordinate to the other? Indeed, had these two historical accidents (discovery of sulfur lithotrophy and iron lithotrophy) occurred in reverse order, we might instead be using the binomial, *Ferrobacillus thiooxidans*.

A biotype phenotypically similar to *T. ferrooxidans*, but lacking ability to oxidize sulfur was isolated by Harrison (1982) and labelled m-1. What could it be: a *Thiobacillus* that has lost the ability to oxidize sulfur?

We must avoid the prejudice which accrues when species names used to commemorate historical accidents describe putative, or outright false, phylogenies. Unfortunately, these names which we use as *symbols for biotype* are two-worded (binomials), thus carry phylogenetic connotations even when we may not wish to make a phylogenetic commitment, yet desire a name as *symbol*.

Mineralogical nomenclature avoids this through the use of mononomial names. On the other hand, inorganic chemical binomials are successful because they cannot be assigned until after the structure they are to represent is fully defined. The "latinized" binomials of bacteria carry a psychological impact which may mislead judgement of the relationships amongst the biotypes they represent. Matters are further complicated when these binomials are "legalized" through the priority rule.

Returning to strain m-1: By means of DNA:DNA homology, m-1 was demonstrated to be unrelated to *T. ferrooxidans*, at least at the genus level (Harrison, 1982). Woese *et al.* (1984), using 16S rRNA catalogs, and Lane *et al.* (1985a), employing 5S rRNA sequencing, were the first to establish lines-of-descent for Thiobacilli. The 5S analysis demonstrated that m-l is more closely related (in terms of "evolutionary distances") to *Chromatium vinosum*, a photosynthetic sulfur bacterium, than to *T. ferrooxidans*. Using the larger 16S rRNA molecule for better phylogenetic resolution, Lane *et al.* (1988) have extended this study, and most biotypes of sulfur and iron oxidizing bacteria have been placed in phylogenetic relationships.

Woese (1987) has described the advantages of rRNA for the determination of phylogenetic relationships. Although other macromolecules (cytochrome, haemoglobin, and enzymes) have been used for the same purpose, their use has been more limited. The present report deals primarily with the *acidophilic* species associated with mineral leaching. The term acidophilic here will refer to those species which grow when inoculated into media posed at $pH \leq 3$. These bacteria are listed in Table 1.

METHODS

Media for cultivating the relevant acidophilic bacteria have been given (Harrison, 1984). Cultures (usually 3- liters) were centrifuged, the pellets washed well in acidic medium lacking energy source, suspended in 0.15 M NaCl, centrifuged a final time, and the resulting pellets were stored at -80 C. From these pellets the RNA was extracted. Cell lysis, RNA extraction and purification are explained elsewhere (Lane *et al.*, 1985b). Sequencing of RNA was by the reverse transcriptase method using three universal 16S rRNA primers already described (Lane *et al.*, 1985b, 1990). The distance method and treeing algorithms used for phylogenetic analyses are also described elsewhere (Olsen, 1988). The lengths of branches on the tree are proportional to the differences in sequences between species, referred to herein as "evolutionary distances".

RESULTS AND DISCUSSION

In Figure 1 (lower, right corner) observe the cluster of acidophiles: DSM 612, *T. thiooxidans*, and *T. ferrooxidans* homology groups. Homology group #3 of T. ferrooxidans is represented by strains F221, ATCC 19859, and ATCC 23270 (type strain). These three strains occupy the same site and are most closely related to *T. thiooxidans*. Homology group #2 is represented by strain Lp. Homology group #4 is represented by strain pH. (See Harrison 1982, 1984, for descriptions of these bacterias.) The "evolutionary distance" between these *T. ferrooxidans* homology groups is about the same as the distance between them and *T. thiooxidans*. Strain DSM 612 is a high mol%G+C "*T. thiooxidans*".

This cluster of acidophilic bacteria appears to from a "natural" group. The relevant strains may be considered different species within a common genus — or different genera within a common family.

Strain m-l, though similar in phenotype, is not closely related to *T. ferrooxidans*, has been demonstrated by Lane *et al.* (1985a) using 5S rRNA sequencing, and by Harrison (1982) using DNA homology. It is more closely related to the photosynthetic purple sulfur bacterium, *Chromatium vinosum* (middle of Figure 1).

"*Leptospirillum ferrooxidans*" (Table 1) is not closely related to any of the Gram negative bacteria in Figure 1, and cannot be placed with precision at the present time. Possibly, is represents a new phylum. Its exact position amongst the Gram negative Eubacteria is presently being determined through further rRNA sequencing.

The moderate thermophiles, strains TH3, Alv, and BCl (Table 1), have 16S rRNA sequences similar to Gram positive bacteria such as *Bacillus* and *Streptomyces*. The Gram positive bacteria have status as phylum (Woese, 1987). The placement of strains TH3, Alv, and BCl relative to this phylum will be discussed elsewhere.

The thermophilic acidophile described by Brierley and Brierley (1973), has yet the be sequenced. Originally named *Sulfolbus brierleyi*, it now bears the binomial, *Acidianus brierleyi*. (See Stetter, 1989). This species is noteworthy for using as energy sources either sulfur, Fe^{++}, or yeast extract, and is undoubtedly a member of the Archaebacteria (Zilling *et al.*, 1980).

Thus, the genes for sulfur and iron oxidation are widely scattered amongst the major phylogenetic groups of bacteria. Do segments of these genes serve only functions in related heterotrophic bacteria? This is an intriguing idea, especially as so many of the lithotrophic bacteria, themselves, also can grow on organic substrates in lieu of sulfur and iron.

Phylogenetic relationships, in addition to their present interest to specialists in taxonomy and evolution, are useful to biochemists and geneticists. Judicious selection of phylogenetically diverse strains which share some common physiological property, for example, iron oxidation, permit an economy of experimentation in the comparative method for establishing metabolic pathways.

Blake (this symposium) has identified different electron transport moieties which apparently are characteristic for particular phylogenetic groups of the iron-oxidizing bacteria.

From the amino acid sequences of these compounds, probes may be constructed and used to reconnoitrer amongst the heterotrophic bacteria related to a lithotroph, searching for base sequences common to the organotroph and lithotroph.

ACKNOWLEDGEMENTS

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Table 1

Acidophilic Bacteria Associated With Mineral Leaching[1]

Strain	Traits	Source & Donor
	Mesophiles. Straight rods.	
Thiobacillus thiooxidans:		
ATCC 19377[2]	S[3]	Libyan sulfur-producing lake. (Amer. Type Cult. Coll.)
Thiobacillus ferrooxidans:		
ATCC 19859	S & Fe[4]	Copper mine, British Col., Canada. (Amer. Type Cult. Coll.)
ATCC 23270[2]	S & Fe	Coal mine effluent, U.S.A. (Amer. Type Cult. Coll.)
F221	S & Fe	Uranium mine water, Austria (K. Bosecker)
Lp	S & Fe	Coal mine, Penna., U.S.A. (P. R. Dugan)
PH	S & Fe	Coal strip-mine, Mo., U.S.A. (B. Jarvis)
m-1	Fe	Coal spoil, Mo., U.S.A. (A.P. Harrison)
	Mesophiles. Curved rods.	
"*Leptospirillum ferroxidans*":		
Z-2	Fe	Armenian copper deposit. (G.A. Zavarzin)
BU-1	Fe	Copper mine drainage, Bulgaria (S.N. Groudev)
Lfla	Fe	Uranium mine water, Mexico (P.R. Norris)
	Moderate thermophiles. Various morphologies.	
Alv	Fe	Coal spoil, Warwickshire, U.K. (P.R. Norris)
BC	Fe	Coal pile, Warwickshire, U.K. (P.R. Norris)
TH3	Fe	Copper mine dump, N.M., U.S.A. (P.R. Norris)

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[1] For additional information concerning these strains, including photomicrographs and references, see Harrison (1986).

[2] Type strain.

[3] S = Elemental sulfur and/or reduced sulfur compounds used as energy source.

[4] Fe = Ferrous iron, as in minerals such as pyrite, used as energy source. In the laboratory commonly cultivated on ferrous sulfate heptahydrate.

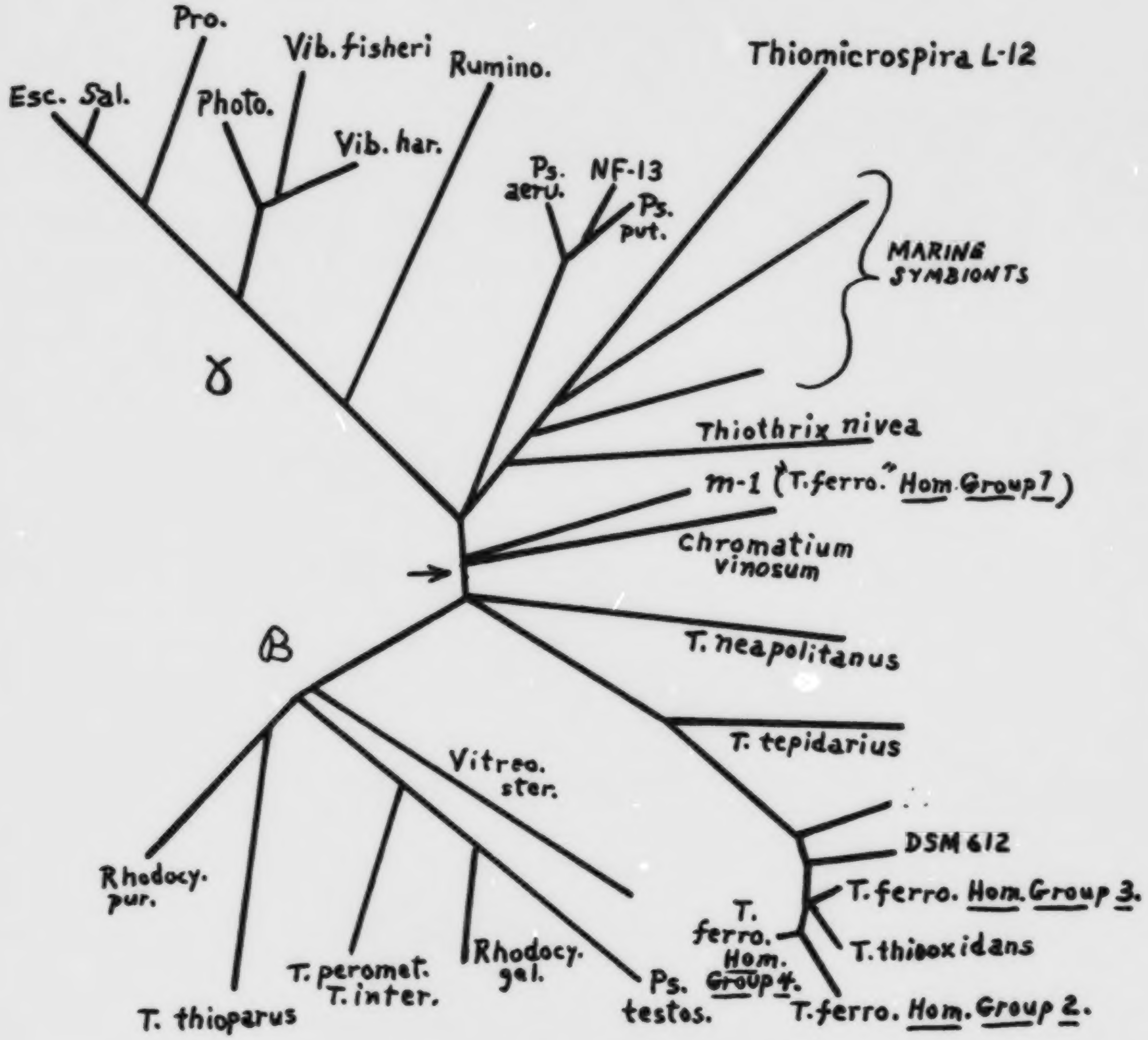
Pro.
Vib. fisheri
Esc. Sal.
Photo.
Rumino.
Vib. har.
Thiomicrospira L-12
Ps. aeru.
NF-13
Ps. put.
MARINE SYMBIONTS
γ
Thiothrix nivea
m-1 (T. ferro." Hom. Group 7)
Chromatium vinosum
→
β
T. neapolitanus
T. tepidarius
Vitreo. ster.
DSM 612
Rhodocy. pur.
T. ferro. Hom. Group 3.
T. ferro. Hom. Group 4.
T. thiooxidans
T. peromet.
T. inter.
Rhodocy. gel.
Ps. testos.
T. thioparus
T. ferro. Hom. Group 2.

Fig. 1 Gram-negative Eubacterial tree, beta and gamma branches only. The arrow indicates where other branches of Eubacteria join. (See Woese, 1987). Observe the *T. thiooxidans*, *T. ferrooxidans* homology groups' (lower, right) and strain m-1 (middle). Abbreviations: Each. = *Escgerichia coli*; Sal. = *Salmonella* ssp.; Pro. = *Proteus vulgaris*; Photo. = *Photobacterium* sp.; Vib. = *Vibrio*; Vib. har. = *Vibrio harveyi*; Rumino. = *Ruminobacter* sp. (= *Bacteroides* sp); Ps. aeru = *Pseudomonas aeruginosa*; NF-13 = un-named marine sulfur-oxidizing sp.; Ps. put. = *Pseudomonas putida*; Marine symbionts = sulfur-oxidizers in symbiotic association with invertrebrates living near oceanic hydrothermal vents. T. = *Thiobacillus*; DSM 612 = un-named acidophile similar to *T. thiobacillus*; Vitreo. ster. = *Vitreoscilla stercoraria*, a filamentous gliding

chemoorganotroph; Ps. testo. = *Pseudomonas testosteroni* , Rhodocy. gel. = *Rhodocyclus gelatinosa*, a purple non-sulfur bacterium; T. peromet. & T. inter. = *Thiobacillus perometabolis* and *T. intermedius* , respectively; Rhodocy. pur. = *Rhodocyclus purpereus* . This tree is modified from Lane et al. (1988).

Enzymology of Respiratory Iron Oxidation

R.C. Blake II, E.A. Shute, and K.J. White

Department of Biochemistry, Meharry Medical College Nashville, Tennessee 37208

ABSTRACT

The current focus in this laboratory is on the identification, isolation and characterization of electron transport proteins responsible for respiratory iron oxidation. Three recent advances in this research are summarized. (i) An *in situ* electrolytic procedure has been developed whereby large quantities of autotrophic iron oxidizing organisms have been achieved for these studies. Up to 230 grams wet weight of *Thiobacillus ferrooxidans* have been harvested from a 55 gallon growth vessel. (ii) Efforts to identify a rusticyanin in cell-free extracts of certain members of the mesophilic *Leptospirillum ferrooxidans* have been unsuccessful. Instead, these organisms express abundant levels of a soluble, acid-stable a-type cytochrome that is reduced directly by sulfatoiron(II). (iii) Efforts to identify either a rusticyanin or an acid-stable cytochrome *a* in cell-free extracts of various members of the moderately thermophilic iron oxidizers have also been unsuccessful. Instead, these latter organisms express generous levels of a novel membrane-bound yellow chromophore that is reduced directly by sulfatoiron(II). A pattern of remarkable diversity in the biochemical mechanisms for respiratory iron oxidation has begun to emerge from these studies.

ENZYMOLOGIE DE L'OXYDATION DU FER AU COURS DE LA RESPIRATION

R.C. Blake II, E.A. Shute et K.J. White

Département de Biochimie, Meharry Medical College
Nashville, Tennessee É.-U.

RÉSUMÉ

Les recherches effectuées dans ce laboratoire portent principalement sur l'identification, l'isolation et la caractérisation des protéines transportants les électrons et responsables de l'oxydation du fer au cours de la respiration. Dans ce domaine, trois percées ont eu lieu récemment. (i) Un procédé électrolytique *in situ* a été mis au point; ce procédé autorise la production en grandes quantités d'organismes autotrophes oxydant le fer. Une cuve de culture de 55 gallons a produit jusqu'à 230 grammes (poids humide) de bactéries *Thiobacillus ferrooxidans*. (ii) Les efforts en vue d'identifier une rusticyanine dans des extraits acellulaires de certains membres de *Leptospirillum ferrooxidans* mésophiles n'ont pas donné de résultats. Toutefois, ces organismes produisent, en grande quantité, un cytochrome de type *a* soluble et stable en milieu acide. Ce cytochrome est directement réduit par le sulfate ferrique (II). (iii) Les efforts en vue d'identifier, dans les extraits acellulaires de différents membres capables d'oxyder le fer, une rusticyanine ou un cytochrome *a* stable en milieu acide ont été vains. Ces derniers organismes produisent cependant de grandes quantités d'un nouveau chromophore jaune entouré d'une membrane; ce chromophore est directement réduit par le sulfate ferrique (II). Ces études ont permis d'entrevoir pour la première fois la remarquable diversité des mécanismes biochimiques en matière d'oxydation du fer au cours de la respiration.

INTRODUCTION

This laboratory is interested in the enzymology of biological respiration on reduced iron and inorganic sulfur compounds. Our experiments over the past several years have concentrated primarily on the identification, isolation and characterization of electron transport proteins responsible for respiratory iron oxidation. Harrison (1986) suggested that the iron oxidizing bacteria could be separated into the following five principal groups: (i) straight rods that oxidize sulfur, defining *T. ferrooxidans;* (ii) straight rods that cannot oxidize sulfur, represented by strain m-1; (iii) curved rods that cannot oxidize sulfur, defining *L. ferrooxidans* ; (iv) facultative, moderately thermophilic eubacteria; and (v) extremely thermophilic archebacteria, represented by Acidianus (*Sulfolbus) sp. T. ferrooxidans* is by far the easiest of the iron oxidizing bacteria to isolate and maintain in culture, and, perhaps for that reason, it has received the most attention in laboratory experiments. Electron transport components that have been implicated in the respiratory oxidation of Fe(II) by *T. ferrooxidans* are indicated in Fig. 1. Cellular components thought to be involved include a blue copper protein called rusticyanin (Cobley and Haddock, 1975), a putative green copper protein (Sugio *et al.*, 1981), two c-type cytochromes (Tikhonova *et al.*, 1967; Ingledew and Cobley, 1980), two *a*-type cytochromes (Ingledew and Cobley, 1980), one or more iron sulfur proteins (Fry *et al.*, 1986; Fukumori *et al.*, 1988), a quinone (DiSpirito *et al.*, 1983), a porin (Kulpa *et al.*, 1986), and a polynuclear Fe(III)-sulfate chelate around the outer cell wall of this Gram-negative bacterium (Ingledew, 1986).

This laboratory recently initiated a systematic survey of the principal electron transport components expressed by members of each of the 5 arbitrary groups of bacteria defined above. The preliminary results of this incomplete, ongoing survey are presented below. Two principal observations are described: (i) rustiycanin is not ubiquitous in cell-free extracts of iron-grown bacteria; and (ii) novel high potential electron transfer components are present in many of the cultures examined.

METHODS

Bacterial culture

The individual bacterial cultures and their sources were as follows: *T. ferrooxidans*, strains 13598, 13661, 14119, 19859, 21834, 23270, and 33020 from the ATCC, strains 5-2al, 6-3al, and TF1, 4, 10, 29, 35, and 35SA from Dr. Olli Tuovinen, Ohio State University, Columbus, OH, and strains A-6, DT-7, FD1, and DEC from Dr. Paul Wichlacz, EG+G Idaho, Inc., Idaho Falls, ID; *L. ferrooxidans*, strain 29047 from the ATCC, strains P3A, R22, and R3 from Dr. Wolfgang Sand, Universitat Hamburg, Hamburg, West Germany, strain z-2 from Dr. Arthur Harrison, Jr., University of Missouri, Columbia, MO; unnamed Gram-negative mesophile, strain m-1 from Dr. Harrison; and unnamed Gram-positive moderate thermophile, strain BCl from Dr. Harrison. Each mesophilic bacterium was grown in batch culture at ambient temperatures in the acidic ferrous sulfate growth medium described by Tuovinen and Kelly (1973) supplemented with 1.6 mM cupric sulfate (Blake and Shute, 1987). Strain BCl, a moderately thermophilic bacterium, was grown in batch culture at 50° C in the above ferrous sulfate medium supplemented with 0.03% yeast extract and 0.01% KCl. Each of the 26 strains was grown in sufficient yield (>5 g wet weight) to permit preliminary identification of the most abundant electron transfer components that could be solubilized by limited sonic oscillation of the cell suspensions at acidic pH. ATCC 23270 was also grown to high yield using an in situ electrolytic procedure described below.

Preparation of cell-free extracts

Cells of each iron-grown bacterium were washed three times with 0.001 N sulfuric acid. The washed cell paste was suspended in 0.01 N sulfuric acid (1:4 g wet cell paste/ml), and the cell suspension was subjected to sonic oscillation for 2 min/g at a power output of 125 watts using an ultrasonic processor manufactured by Heat System-Ultrasonics, Inc. Care was taken to maintain the temperature of the solution below 7°C. Large cell debris was removed by centrifugation of the sonicate at 10,000 x g for 10 minutes. Further centrifugation of the low speed supernatant at 20,000 x g for 120 minutes yielded a pellet and a slightly cloudy supernatant. The normally highly colored supernatant was then subjected to ammonium sulfate precipitation to separate the different colored fractions.

Absorbence measurements

Absorbance spectra were obtained on a Cary 14 dual-beam spectrophotometer rebuilt and modified by On Line Instrument Systems, Inc., Jefferson, GA. Instrument control and data analysis were accomplished *via* a Zenith ZF118 computer interfaced to the rebuilt spectrophotometer.

RESULTS

Scope of present experiments

We began our survey of the principal cellular components responsible for respiration on iron with two factors in mind. First, like all chemolithotrophs that utilize substrates of high reduction potential (Aleem *et al.*, 1963), each iron-oxidizing organism required a high throughput of Fe(II) to obtain sufficient energy for growth. This requirement necessitates that the relevant electron transfer components be expressed in high yield during growth on Fe(II). We thus focused our attention on redox-active biomolecules that appeared to be present in iron- grown cells in relative abundance. Second, it is generally accepted that the initial electron transfer reaction from Fe(II) to a cellular component must occur outside the plasma membrane in all acidophilic iron oxidizers (Ingledew, 1982; Ehrlich, 1981; Cobley and Cox, 1983; Hooper and DiSpirito, 1985). We therefore concentrated our initial search for relevant redox-active biomolecules at two values of pH. Electron transfer components that proved to be stable in cell-free extracts prepared at pH 2.0 were examined for their possible role(s) in the initial portion of the respiratory chain, while electron transfer components that proved to be stable in extracts prepared at neutral pH were examined for their possible role(s) in the terminal portion of the chain. The results of our preliminary survey on the former, acid-stable components are summarized here.

Many Gram-negative mesophiles contain rusticyanin

Rusticyanin is an acid-stable, type I copper protein that may constitute as much as 5% of the total soluble protein synthesized by cells of *T. ferrooxidans* that have been grown autotrophically on Fe(II) (Cox and Boxer, 1978). The purified protein has a reduction potential of 680 mV (Ingledew and Cobley, 1980) and can be reduced directly by Fe(II). Furthermore, the synthesis of rusticyanin is repressed when *T. ferrooxidans* is grown solely on reduced sulfur compounds and induced when such sulfur-grown cells are subsequently exposed to soluble iron (Kulpa *et al.*, 1986; Jedlicki *et al.*, 1986). These observations are compelling evidence in support of the hypothesis that rusticyanin must be an important component of the iron respiratory chain.

A rusticyanin-like protein was identified in 22 of the 26 cultures examined. The rusticyanin-positive cultures comprised all 19 cultures identified as *T. ferrooxidans*, strain m-1, and two putative cultures of *L. ferrooxidans*, ATCC 29047 and z-2. In most cases, the supernatant obtained after centrifugation of the sonicated cell suspension contained sufficient oxidized rusticyanin to impart a distinct and striking blue hue to the cell- free extract. The supernatant derived from strain m-1 did not exhibit this blue hue until the cell-free extract had been incubated overnight at 4°C with an excess of ferric ions. In all 22 cases, the soluble rusticyanin in the supernatant precipitated in the 45 to 95% saturated ammonium sulfate fraction. Dissolution of each blue precipitate in 0.01 N sulfuric acid produced a homogeneous solution with a visible absorbance spectrum characteristic of a type I copper protein (data not shown).

The exact role that the rusticyanin plays in the iron- dependent electron transport scheme is unclear. The principal objection to the hypothesis that rusticyanin is the primary oxidant of ferrous ions in the *T. ferrooxidans* electron transport chain is that the electron transfer reactions monitored in vitro between sulfatoiron and rusticyanin are far too slow to account for the facile Fe(II)-dependent reduction of molecular oxygen in the intact organism (Lewis *et al.*, 1983; Lappin *et al.*, 1985; Blake and

Shute, 1987). Nonetheless, the presence of abundant levels of rusticyanin in so many acidophilic iron oxidizing organisms, particularly those like m-1 that do not oxidize reduced sulfur compounds, strengthens the suspicion that rusticyanin is an important participant in the iron respiratory chain of those bacteria that express it.

Some Gram-negative mesophiles do not contain rusticyanin

Not all Gram-negative mesophiles that respire on Fe(II) contain detectable levels of rusticyanin. Cell-free extracts derived from each of the 3 strains of *L. ferrooxidans* obtained from Dr. Sand appeared to contain no detectable rusticyanin whatsoever. What these iron-grown bacteria did express in considerable abundance was the acid-stable, soluble cytochrome shown in Fig. 2. Fig. 2 shows the absolute visible spectra of the oxidized (trace a) and the Fe(II)-reduced (trace b) cytochrome from *L. ferrooxidans* strain P3A. The absorbance spectrum of the oxidized cytochrome was determined in 0.01 N sulfuric acid at 25° C. The absorbance spectrum of the reduced cytochrome was recorded immediately after a single crystal of reagent grade ferrous sulfate had dissolved in the cuvette. The inset is a difference spectrum representing the spectrum of the reduced cytochrome minus that of the oxidized cytochrome. The peaks of the difference spectrum lie at 442 and 579 nm. These spectral features are characteristic of a-type cytochromes. The spectra shown in Fig. 2 are representative of the proteins derived from each of the three strains of *L. ferrooxidans*, P3A, R22, and R3, that contained high levels of this cytochrome.

The *a*-type cytochrome was substantially purified by ion exchange chromatography on CM- and DEAE-Sephadex at pH 5.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on the highly purified *a*-type cytochrome showed one major band at around 12,000 daltons with a minor band on either side. The highly purified cytochrome was reduced rapidly with low concentrations of sulfatoiron(II). Since a rusticyanin-like protein could not be demonstrated in these organisms, the working hypothesis is that these 3 mesophiles express a quite different set of high potential respiratory chain components responsible for autotrophic growth on ferrous ions.

It should be noted that two other strains identified as *L. ferrooxidans*, ATCC 29047 and z-2, contained abundant levels of rusticyanin. We have since learned that the identity of ATCC 29047 may be questionable (A. Harrison, pers. commun., 1989). In view of the results just presented, it would be prudent to also question the purity and identity of our transplant of strain z-2.

Gram-positive moderate thermophiles contain a novel yellow chromophore

One Gram-positive moderately thermophilic iron oxidizer, designated as strain BCl, was grown in sufficient quantity to permit an examination of its cytochrome and/or colored electron transport protein content. No evidence for a rusticyanin-like molecule could be detected in cell-free extracts of iron-grown BCl. Although membrane-associated cytochromes were evident in the pellet obtained after prolonged sonic oscillation of strain BC, the amounts of such cytochromes were far below those expressed by the *L. ferrooxidans* described above. What strain BCl did produce in copious amounts was a novel yellow material whose spectral properties are featured in Fig. 3. The supernatant derived from prolonged sonic oscillation of strain BCl was a deep yellow color. The yellow-colored material precipitated in the 45 to 95% saturated ammonium sulfate fraction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on the yellow material in the ammonium sulfate pellet showed only two bands of equal staining intensity at around 8,000 and 12,000 daltons, respectively.

Fig. 3 shows the absolute visible spectra of the oxidized (trace *a*) and the Fe(II)-reduced (trace *d*) yellow chromophore. The absorbence spectrum of the oxidized yellow material was determined in 0.01 N sulfuric acid at 25° C. The remaining absorbence spectra were obtained by repeatedly scanning the reaction mixture after a single crystal of reagent grade ferrous sulfate had dissolved in the cuvette. The spectrum identified by the d was determined 5 minutes after the addition of the reducing agent. The *inset* is a difference spectrum representing the spectrum of the reduced yellow chromophore minus that of the oxidized yellow chromophore. The peak of the difference spectrum lies at 458 nm. The current working hypothesis is that the Gram-positive moderate thermophiles express yet another set of high potential respiratory chain components during facultative growth on ferrous ions. The yellow chromophore may represent a new enzyme cofactor unique to resiratory iron oxidation in these moderate thermophiles.

In situ electrolysis shows great promise for large scale culture of bacteria that respire on Fe(II)

The problem of obtaining a sufficient mass of iron-grown bacteria for subsequent biochemical studies has recently been addressed using a novel means of achieving the large scale cultivation of these organisms. Several laboratories have reported that enhanced yields of iron oxidizing bacteria may be achieved by *in situ* electrolysis of the soluble iron in the growth medium (Kinsel and Umbreit, 1964; Kovrov *et al.*, 1978; Denisov *et al.*, 1980; Yunker and Radovich, 1985). Accordingly, an *in situ* electrolysis apparatus was assembled in which the soluble iron substrate in the growth medium was subjected to continual electrochemical reduction at the cathode. The actual electrochemical apparatus to achieve enhanced yields of iron oxidizing bacteria is diagrammed schematically in Fig. 4. The principal component of this apparatus is the electrolytic flow cell, available commercially from ElectroCell AB, Sweden. The catholyte, which contained the soluble iron and the viable bacteria, was pumped at a flow rate of approximately 1 gallon/minute through a fine mesh of graphite felt that served as the cathode. A very high surface area was achieved at the cathode by forcing the solution to percolate through the network of the felt. The anolyte (3.0 N sulfuric acid) was pumped at a similar rate past the platinum electrode that constituted the anode. The Nafion membrane that separated the two chambers permitted protons to pass from the anolyte to the catholyte concomitantly with electron flow. The digital coulometer permitted quantification of the current passed.

The relevent chemistry in the electrochemical apparatus is illustrated schematically in Fig. 5. Ferric ions produced as a consequence of bacterial energy metabolism are continually reduced back to the ferrous state at the cathode; electrons for this reduction are derived from the oxidation of water at the anode. Meanwhile, protons consumed during the bacterial-dependent reduction of oxygen are replaced by protons that cross the semipermeable barrier as a consequence of current flow; the semipermeable membrane thus replaces the more traditional salt bridge. The iron-oxidizing bacteria in the catholyte thus experience a constant supply of electrons and may continue to grow until some other factor in the medium becomes limiting.

The *in situ* electrolysis procedure outlined above was applied to *T. ferrooxidans* ATCC 23270. The catholyte consisted of 55 gallons of viable *T. ferrooxidans* in the acidic ferrous sulfate growth medium described above. When bacterial growth in the 55 gallon vessel became so abundant that the electrochemical apparatus could no longer achieve the complete reduction of the entire solution, the culture was harvested. A total of 230 grams of wet cell paste of *T. ferrooxidans* was achieved from one growth. This yield represents greater than a 25-fold increase in cell mass/volume over that typically achieved in batch culture without electrolysis. Furthermore, the electrolyzed bacterial cells were indistinguishable from normal batch-grown cells in both their rusticyanin and their cytochrome contents. It is anticipated that all organisms that obtain energy from the aerobic oxidation of Fe(II) will benefit from a continuous supply of the reduced substrate and that *in situ* electrolysis techniques will result in greatly increased ratios of cell mass/unit volume for all iron oxidizing bacteria examined.

CONCLUSIONS

It is evident from the diversity of the organisms that comprise the 5 principal taxonomic groups discussed above that acidophilic iron oxidizers do not share a unique line of descent. Since species capable of iron oxidation are genomically so diverse, conclusions regarding the biochemical pathway(s) for iron oxidation derived from one species or strain cannot necessarily be extrapolated to another species or strain. Indeed, the current data suggest that the individual iron oxidation respiratory chains may be arbitrarily divided into the following 3 categories:

(i) Those that contain rusticyanin. Organisms that express rusticyanin include all of the strains of *T. ferrooxidans* examined thus far (19 total), *L. ferrooxidans* ATCC 29047 and z-2, and the strain designated as m-1.

(ii) Those that contain a redox-active, acid-stable, soluble, possibly a-type, cytochrome. The 3 strains of *L. ferrooxidans* that expressed this cytochrome did not express a rusticyanin-like molecule when grown autotrophically on ferrous ions.

(iii) Those that contain an acid-stable membrane protein bearing a novel yellow chromophore. This yellow chromophore has been detected in one strain of the Gram-positive moderate thermophiles grown at 50°C on ferrous ions.

The availability of a stable of organisms that share a fundamentally common pathway of iron respiration may provide opportunities to study a particular portion of the pathway in some of the members that proves to be intractable in others. A key protein in the pathway that is extremely difficult to isolate from one organism may prove to be easily obtained in good yield from another. On the other hand, detailed comparisons among the groups of iron oxidizers that express different mechanisms and pathways may provide the opportunity to deduce the advantages and disadvantages of each. In terms of the eventual application of this basic knowledge to biohydrometallurgical problems, it should be emphasized that each of the iron oxidizing organisms examined in this study was originally isolated from a mining environment. Although *T. ferrooxidans* is the most extensively characterized member of this group of bacteria, that does not mean that it will prove to be the predominant or most useful organism in any or all of the eventual biohydrometallurgical processes that may be developed. The need for basic information regarding the energy metabolism of all of these organisms is evident.

ACKNOWLEDGMENTS

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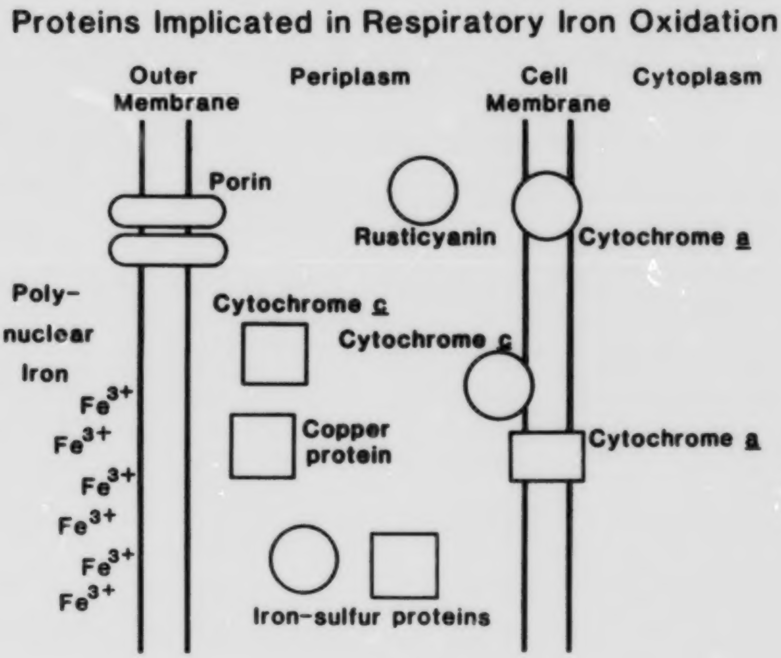
Proteins Implicated in Respiratory Iron Oxidation
Outer Membrane
Periplasm
Cell Membrane
Cytoplasm
Porin
Rusticyanin
Cytochrome a
Poly-nuclear Iron
Fe3+
Fe3+
Fe3+
Fe3+
Fe3+
Fe3+
Cytochrome c
Cytochrome c
Copper protein
Cytochrome a
Iron-sulfur proteins

Fig. 1 Proteins implicated in respiratory iron oxidation by *T. ferrooxidans*

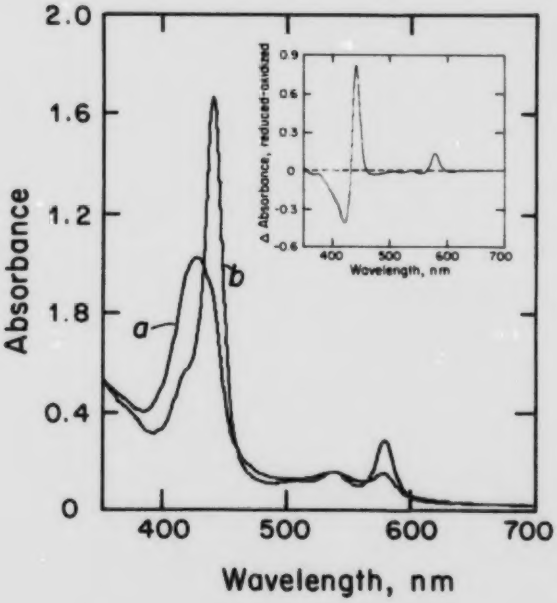

Fig. 2 Acid-stable cytochrome in *L. ferrooxidans*

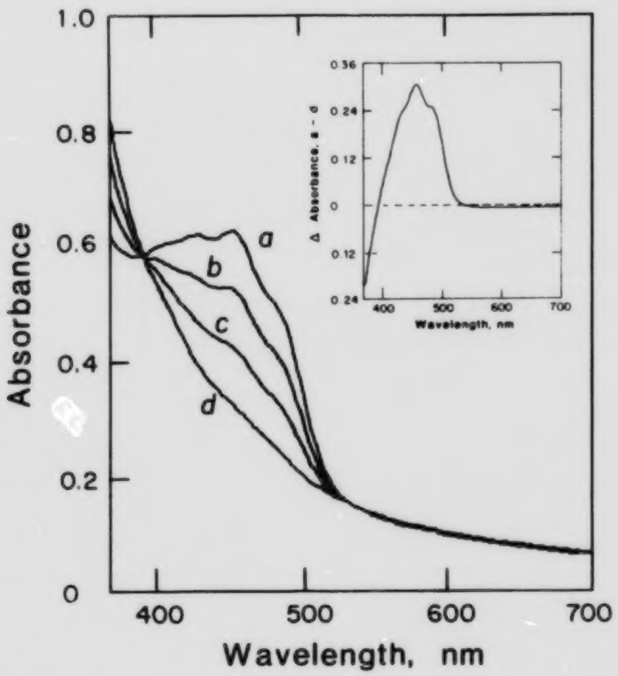

Fig. 3 Acid-stable yellow chromophore in strain BC

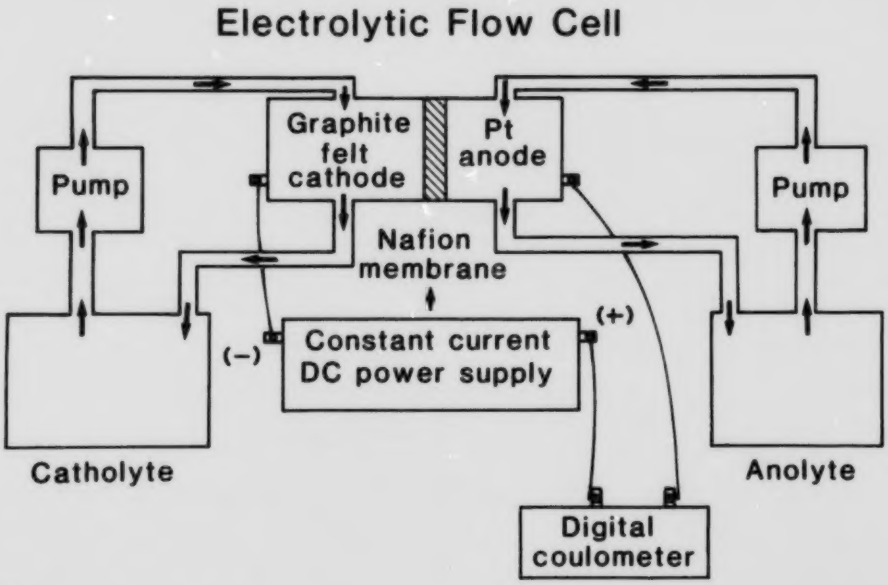
Electrolytic Flow Cell
Pump
Graphite felt cathode
Pt anode
Pump
Nafion membrane
(–)
Constant current DC power supply
(+)
Catholyte
Anolyte
Digital coulometer

Fig. 4 *In situ* electrolysis apparatus

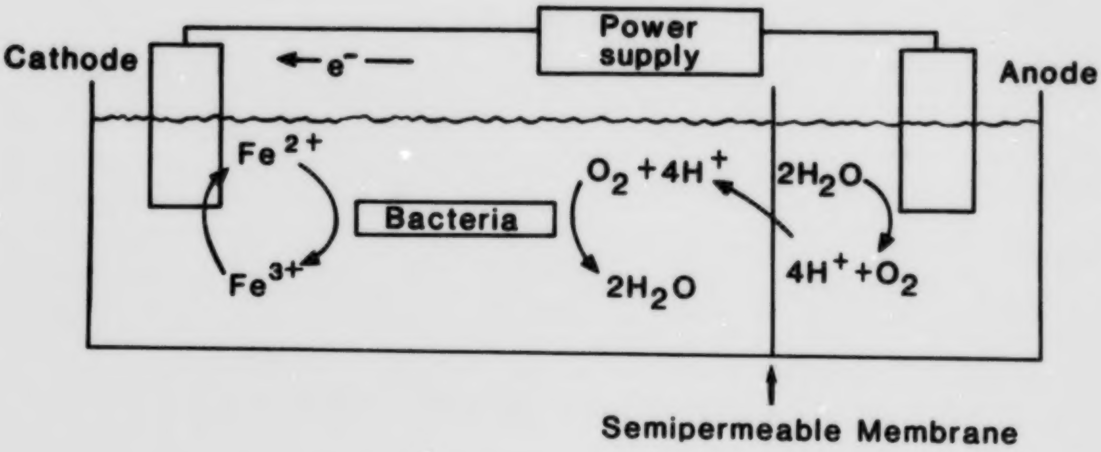
Power supply
Cathode
Anode
e^-
Fe^{2+}
Fe^{3+}
Bacteria
$O_2 + 4H^+$
$2H_2O$
$2H_2O$
$4H^+ + O_2$
Semipermeable Membrane

Fig. 5 Chemistry of *in situ* electrolysis

ISOLATION OF NOVEL ACIDOPHILES AND THEIR POTENTIAL USE IN BIOLEACHING OPERATIONS

D.B. Johnson, M.F. Said, M.A. Chauri and S. McGinness

School of Biological Sciences, University of Wales Bangor, Gwynedd. U.K.

SUMMARY

Using newly-developed solid media for growing acidophilic bacteria it has been possible to isolate a range of iron-oxidising and heterotrophic bacteria, displaying both physiological and behavioural diversity. Isolates were divided into five groups: two of iron-oxidising chemolithotrophs (*Thiobacillus*-like and *Leptospirillum*-like), mixotrophic iron-oxidisers, heterotrophic iron-oxidisers and non iron-oxidising heterotrophs. Some heterotrophic isolates were able to reduce ferric iron, and patterns of iron-cycling were observed in some mixed cultures. The presence of heterotrophic bacteria in glucose-supplemented pyrite cultures retarded pyrite oxidation, due to the reduction of ferric iron; however, in the absence of glucose, a mixed culture of *L. ferrooxidans* and a heterotroph proved to be the most efficient pyrite leaching system tested. Iron oxidisers varied in their attachment to pyrite, glass or ferric hydroxysulfate, and in their tolerance of heavy metals.

ISOLATION DE NOUVELLES BACTÉRIES ACIDOPHILES ET L'UTILISATION POSSIBLE DE CES BACTÉRIES POUR LA BIOLIXIVATION

D. B. Johnson, M.F. Said, M.A. Ghauri et S. McGinness

École de Sciences Biologiques, Université de Whales Bangor, Gwynedd U.K.

RÉSUMÉ

À partir d'un milieu de culture nouvellement conçu pour la croissance des bactéries acidophiles, il a été possible d'isoler une variété de bactéries capables d'oxyder le fer dont plusieurs hétérotrophes. Ces bactéries présentent des différences physiologiques ainsi que des différences de comportement. Des isolats ont été divisés en cinq groupes constitués comme suit : deux types de bactéries chimiolithotrophes oxydant le fer (du genre *Thiobacillus* et du genre *Leptospirillum)*, des bactéries mixotrophes oxydant le fer, des bactéries hétérotrophes oxydant le fer et des bactéries hétérotrophes n'oxydant pas le fer. Certains isolats ont été capables de réduire les ions ferriques et on a observé une modification cyclique du fer dans certaines cultures. La présence de bactéries hétérotrophes dans des cultures auxquelles on a ajouté du glucose, a retardé l'oxydation de la pyrite en raison de la réduction des ions ferriques. En l'absence de glucose toutefois, c'est une culture mixte constituée de *L. ferrooxidans* et d'une bactérie hétérotrophe qui s'est révélée la plus efficace pour la lixiviation de la pyrite. Les bactéries oxydant le fer ont montré des affinités différentes pour la pyrite, le verre et l'hydrosulfate ferrique ainsi qu'une tolérance aux métaux lourds différente.

INTRODUCTION

It has become apparent in the last decade or so that highly acidic (pH <3) metalliferous environments may be populated by a range of microorganisms other than bacteria of the genus *Thiobacillus*. Although *T. ferrooxidans* is the most well- known iron-oxidising acidophile, others such as *Leptospirillum ferrooxidans* (Harrison and Norris, 1985) and undesignated coccoid bacteria (Huber *et al.*, 1986) have been isolated in pure culture. The '*T. ferrooxidans*' label itself has been shown to include bacterial strains of wide genomic diversity (Harrison, 1986).

Acidophilic heterotrophic bacteria, principally *Acidiphilium* spp., have been isolated both from mine waters and supposedly pure cultures of *T. ferrooxidans* (e.g. Harrison, 1981; Wichlacz and Unz, 1981; Johnson and Kelso, 1983). These are dominantly oligotrophic, Gram-negative, obligately aerobic rods, incapable of ferrous iron oxidation, though sulphur may be oxidised in organic media (Harrison, 1981).

Most acidophilic *Thiobacilli* are obligate chemolithotrophs, incapable of growing in organic media. *T. acidophilus* is, however, a facultative autotroph, growing on elemental sulphur or simple organic compounds such as glucose (Guay and Silver, 1975). Mixotrophic growth has been demonstrated in a strain of *T. ferrooxidans* (Barros *et al.*, 1984) though, in contrast to *T. acidophilus*, this bacterium was incapable of growth in straight organic media.

Isolation of acidophiles, particularly chemolithotrophs, has often been hindered by a lack of solid media capable of promoting their growth. A major drawback to the frequently-used methods of enrichment cultures and serial dilutions is that the bacteria isolated tend to be those which grow most rapidly in synthetic media in laboratory conditions — this tends, for example, to favour strains of *T. ferrooxidans* over *L. ferrooxidans*. Significant indigenous species and strains may therefore have been overlooked.

This paper describes a method for the direct isolation of acidophilic bacteria from environmental samples, and describes the diversity of autotrophic and heterotrophic microorganisms which have thus far been isolated. Their potential in microbial leaching operations is also discussed.

MATERIALS AND METHODS

Sources of bacteria

Acidophilic bacteria were isolated principally from acid waters from disused metalliferous mines in North Wales, U.K. Pure and mixed cultures, sent to the authors by various colleagues, were also used as source materials. Other bacteria were obtained from the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland.

Liquid media

Iron-oxidising bacteria were grown in ferrous sulfate media (10-50mM), pH 1.8. Heterotrophic acidophiles were grown in 10mM glucose or 0.025% (w/v) tryptone soya broth (TSB) media, pH 2.0-2.5 (Johnson *et al.*, 1987; iron oxidation and reduction were monitored by adding ferrous or ferric sulfate at 25mM, and titrating culture aliquots against 1mM $KMn0_4$ in sulfuric acid.

Pyrite cultures were prepared with ground (Cae Coch sulfur mine (Johnson *et al.*, 1979) using the basal salts medium of McCready *et al.*, (1986). A 1% suspension (w/v) of the ore (which contained 82% FeS_2) was used, and some cultures were supplemented with 10mM (final concentration) glucose. Pure cultures (*T. ferrooxidans* NC1B 11820 and an *L. ferrooxidans*isolate 'SJ1') and mixed cultures (with an *Acidiphilium* — like isolate 'SJH') were established, and were incubated at 30°C, unshaken. Total soluble iron was estimated by atomic absorption spectrophotometry.

Solid media

The basic medium used was the FeTSB medium described by Johnson *et al.*, (1987). This was modified by introducing a heterotrophic isolate (SJH) into molten (45°C) medium, which was poured as a thin layer and overlaid with sterile FeTSB. Heterotroph SJH was pregrown in 10mM galactose/0.25%TSB/25mM ferrous sulfate, and added at 5% (v/v). The modified medium (FeTSBo) supported growth of all strains of mesophilic and moderately thermophilic bacteria tested. Other variations of FeTSB included reducing the ferrous iron concentration to 0.1% w/v (for universal heterotrophic growth) and replacing ferrous with ferric sulfate.

Carbon dioxide fixation and glucose utilisation

The ability of acidophiles to fix CO_2 was tested by growing cultures in the presence of $^{14}CO_2$ (1ml of 0.5M $NaHCO^3$ per 100 ml culture at a specific activity of 20,000 CPM/umol). Glucose utilisation was tested by adding 1mM (final concentration) U-^{14}C-glucose, at a specific activity of 90,000 CPM/umol. Incorporation of ^{14}C was determined by filtering samples (2ml) through 0.2μm Whatman membrane filters (Eccleston *et cl.*, 1985).

Adsorption to surfaces

The ability of iron-oxidising acidophiles to attach to pyrite, glass and ferric hydroxysulfate was tested. For pyrite and glass, this entailed adding ground pyrite or glass beads (210μm diameter) to suspensions of bacteria in pH 2.0 basal salts, and counting numbers of viable, unattached bacteria over 100 mins., against a control. The surface area of both pyrite and glass was 780cm^2/100ml culture. Absorption to ferric hydroxysulfate, which precipitates out in iron cultures of pH >2.2 was monitored by growing cultures at (initial) pH levels of 1.5 and 2.0 and noting differences in viable counts as oxidation progressed. Since no precipitate formed at the lower pH, this was taken as the control.

Metal tolerance

Tolerance of iron-oxidising to some metals (copper, molybdenum, uranium and silver) was tested by growing isolates in 10mM ferrous sulfate containing differing metal concentrations. For acidophilic heterotrophs, tests were performed on solid media (which correlated well with liquid media), using reduced iron FeTSB, and tolerance to ferrous and ferric iron, and zinc, was also assessed.

RESULTS

Diversity of acidophiles

Acidophilic microorganisms were readily classified into iron-oxidising acidophiles and heterotrophic isolates, depending on whether or not colonies were ferric iron-encrusted on FeTSB and FeTSBo plates. The latter were far superior in terms of numbers and diversity of iron-oxidising isolates, and the length of time taken for them to appear (5-20days, depending on species and strain). Iron-oxidizing isolates were subdivided on the basis of colony size and morphology, and latterly on cell characteristics. Three distinct cell forms were identified: small curved, motile rods, small straight rods, and larger straight rods which tended to form filaments of 10-30 cells; these groups also displayed low rates of CO_2 fixation (e.g. 3.42 μg C ml^{-1} for isolate OP14 grown in 50mM $FeSO_4$ compared with 5.27 μg C ml^{-1} for *T. ferrooxidans* (NCIB 11820)) and this did not correlate with biomass C, obtained from measuring cell size and bacterial numbers (8.80 μg C ml^{-1}). It is possible that this group of iron oxidisers is mixotrophic; e.g., addition of 0.02% (w/v) casein hydrolysate increased cell numbers from 1.43 to 1.88 x 10^{7}ml^{-1}, compared with unamended cultures (counted at the end of ferrous oxidation). However, the evidence is somewhat ambiguous, as CO_2 fixation was not suppressed by addition of casein hydrolysate, and the isolates (OP14 and SF10) did not display any greater uptake of glucose than the type strain (NC1B and 11820) of *T. ferrooxidans*.

Two isolates (one from Cae Coch -CCH7, the other from an Australian coal mine-M4Lhet) showed different growth characteristics from both main-stream iron-oxidising and heterotrophic acidophiles on solid media, growing initially as gelatinous, unstained, typically heterotrophic colonies, but latterly developing ferric- staining. The isolates were purified by repeated subculturing and single colony isolation. In liquid media, both bacteria oxidised ferrous iron (though at a slower rate than *T. ferrooxidans*) and both were able to be subcultured successfully through ferrous iron-free media; the isolates were unable to fix CO_2 (Table 1) and they were classed as a separate group of heterotrophic acidophiles (Group IV). Morphologically, they were similar in being Gram-negative rods, characteristically growing as filaments, though this was more pronounced with isolate CCH7, where the filaments resembled those produced by sheath-forming *Leptothrix* spp. Non iron-oxidising heterotrophs were classified separately (Table 1).

Table 1

Some characteristics of acidophilic, mesophilic bacteria

	Cell morphology	Fe^{2+} oridation	S^0 oxidation	Co_2 fixation	Gluose utilisation	Copper tolerance μM	Uranium tollerance μM	Notes
Group I	curved rods c.1.o x 0.3 μm	+	-	+	-	1-5	2	*L. ferrooxidans* like bacteria
Group II	straight rods c.1.2 x 0.6 μm	+	+	+	-	100-200	<	*L. ferrooxidans* like bacteria
Group III	filamentous rods c.4.5 x 0.6 μm	+	(+)	+	-	5-10	2	Filamentous Fe^{2+} oxidisers
Group IV	filamentous rods c.3.5 x 0.6μm	+	-	-	+	<1	<0.5	Fe^{2+} oridising heterotrophs
Group V	straight rods c.1.2 x 0.5μm	-	-	-	+	1-50	<0.5	*Acidiphilium* like bacteria

Ferric iron reduction

Of 32 heterotrophic isolates tested, 16 displayed an ability to reduce ferric iron when grown on ferric iron—glucose plates. Rates of reduction varied (data not shown), the most pronounced being shown by isolate SJH. In mixed cultures with *T. ferrooxidans*, a sequence of iron cycling was observed, in which an oxidation phase was followed by complete reduction to the ferrous form, which was in turn reoxidised after a period of about 6 days (Figure 1). The mechanism of ferric reduction in SJH is, as yet, unproved, though it does appear to be enzymic: cell- free, stationary phase cultures will not reduce, reduction is halted by hearing cultures to 70°C for 15 mins., and no extracellular organic acids were detected.

Pyrite oxidation in pure and mixed cultures

T. ferrooxidans and *L. ferrooxidans* showed significant differences in response to growing in the presence of glucose and in mixed culture with heterotroph SJH (Figures 2 and 3). *T. ferrooxidans* was not affected by 10mM glucose, and pyrite oxidation was not stimulated by the presence of the heterotroph in glucose-free cultures. However, mixed cultures with glucose were considerably less efficient at oxidising pyrite, presumably due to heterotrophic ferric reduction diminishing the oxidation of the mineral by this cation (the 'indirect' mechanism). The same effect was also seen in pyrite-glucose mixed cultures with *L. ferrooxidans*. In contrast, however, this bacterium was completely inhibited by the presence of 10mM glucose (numbers fell to 2 ml^{-1}); even though total soluble iron (entirely in the ferrous form) increased. The mixed culture of *L. ferrooxidans* and SJH, in the absence of glucose, was the most efficient pyrite oxidation system of all those tested.

Attachment to surfaces

Iron-oxidising acidophiles displayed considerably different affinities for solid surfaces. Isolate OP14 (a Group III isolate) showed the strongest binding to pyrite with >99% of bacteria attaching to the mineral within 10 mins (Figure 4). A *L. ferrooxidans* isolate (SY) was most readily adsorbed on glass beads, though binding of all strains was less than that on pyrite. Isolate SJ2 (a Group II bacterium) was most readily adsorbed on ferric hydroxysulfate (Figure 5); in pH 2 cultures, numbers of this bacterium initially increased, but fell dramatically on formation of the precipitate, in contrast to the pH 1.5 culture and other isolates at pH 2.

Metal tolerance

In general, the highest tolerance to heavy metals was shown by the *T. ferrooxidans* strains (Group II). However, uranium tolerance was significantly greater in Groups I and III. The iron-oxidising heterotrophs (Group IV) were relatively intolerant of heavy metals, often more so than Group V heterotrophs; e.g., ferric iron was toxic at only 25mM. Heterotrophic microorganisms displayed a range of tolerance to heavy metal. One isolate (an yeast-MZY-therefore outside the present classification system) showed 100-fold greater tolerance to uranium and 20-fold to silver than the most tolerant acidophilic heterotrophic bacterium.

CONCLUSIONS

It is apparent that a considerable diversity of acidophilic microorganisms exists in natural and man-made environments, and that the full biotechnological potential of these acidophiles has not thus far been realised. The simple classification system presented herein has its limitations; e.g., it excludes thermophilic species, sulfur- oxidisers such as *T. thiooxidans*, and has no genetic support. Nevertheless, as an empirical system, it has proved very useful in our laboratory, and it has a rational base – Group III bacteria have characteristics in common with Groups II and IV; Group IV with Groups III and V etc.. The division of iron-oxidising acidophiles into four groups may seem overly complex, though Harrison (1986) has stressed the genetic diversity of these bacteria. Isolates classified as Groups III and IV have not been described in detail hitherto, though Group III bacteria could be related to the mixotrophic *T. ferrooxidans* strain described by Barros *et al.*; Wakao *et al.*, (1984) have reported the presence of filamentous iron-oxidising bacteria in acid streamers. Some non-iron-oxidising bacteria used in this work (e.g. isolate PSF) were sufficiently different from typical *Acidiphilium* supp. to suggest subdividing Group V further.

There are indications that some of the isolates described herein may be of considerable potential in both metal mobilisation and recovery operations. Greater uranium tolerance may, for example, enable Groups I and III to be the most effective leaching bacteria in uranium extraction. Systems which utilise iron-oxidising bacteria immobilised on surfaces (e.g. Grishin and Tuovinen, 1988) may be more efficient with readily-attached strains, and since this varies with support matrix materials, different strains may need to be selected. Mixed cultures of iron-oxidising chemolithotrophs and iron-reducing heterotrophs (such as SJH) have contrasting efficiencies at pyrite oxidation. In the presence of an organic source, the reduction process causes a reduced rate of mineral leaching by lowering the concentration of the ferric oxidant; but in the absence of exogenous organic matter, the efficiency of an organic-sensitive iron-oxidiser, such as *L. ferrooxidans*, is dramatically improved, presumably by the scavenging activities of the heterotroph (i.e. the same mechanism that promotes growth of *L. ferrooxidans* on FeTSBo plates). The potential of isolate SJH for reducing, rapidly and comprehensively, ferric to ferrous iron provides an additional control mechanism for modifying the ionic composition of leaching solutions and acid mine waters in general.

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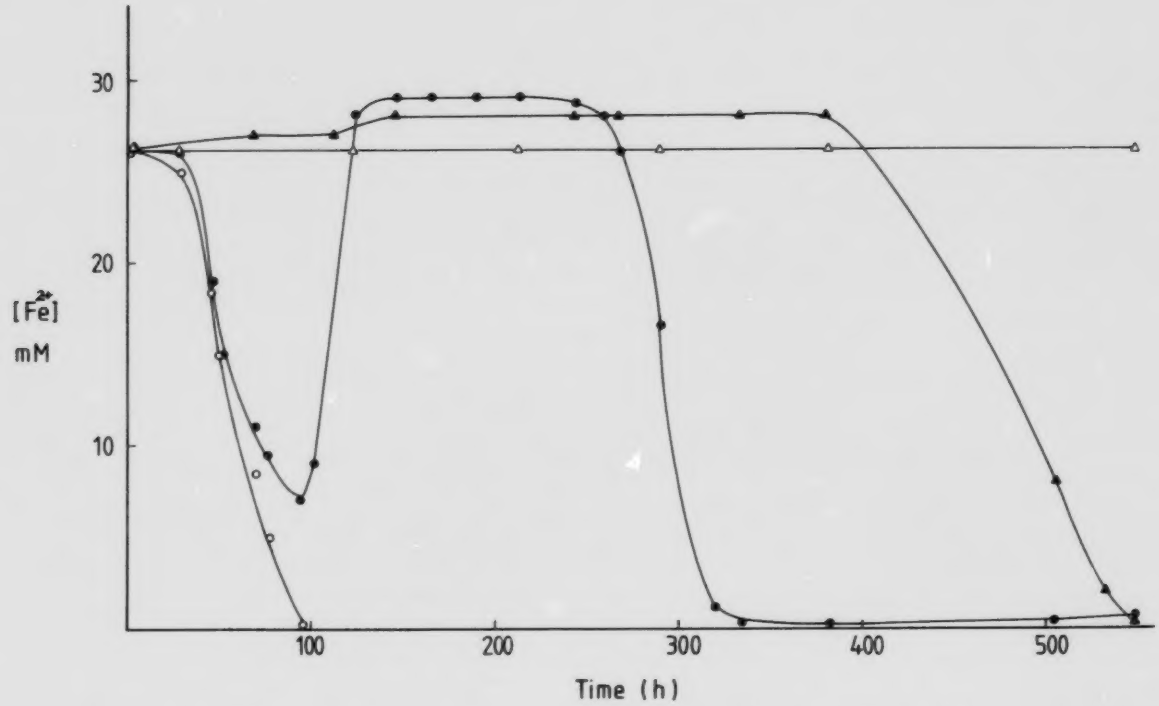

Fig. 1 Iron transformations in pure and mixed cultures of acidophilic bacteria, grown in 25mM ferrous sulfate – 10mM glucose medium. ○ *T. ferrooxidans (NCIB 11820);* ● *T. ferrooxidans + heterotroph SJH;* △ *L. ferrooxidans* (isolate SJ1); ▲ *L. ferrooxidans* + SJH.

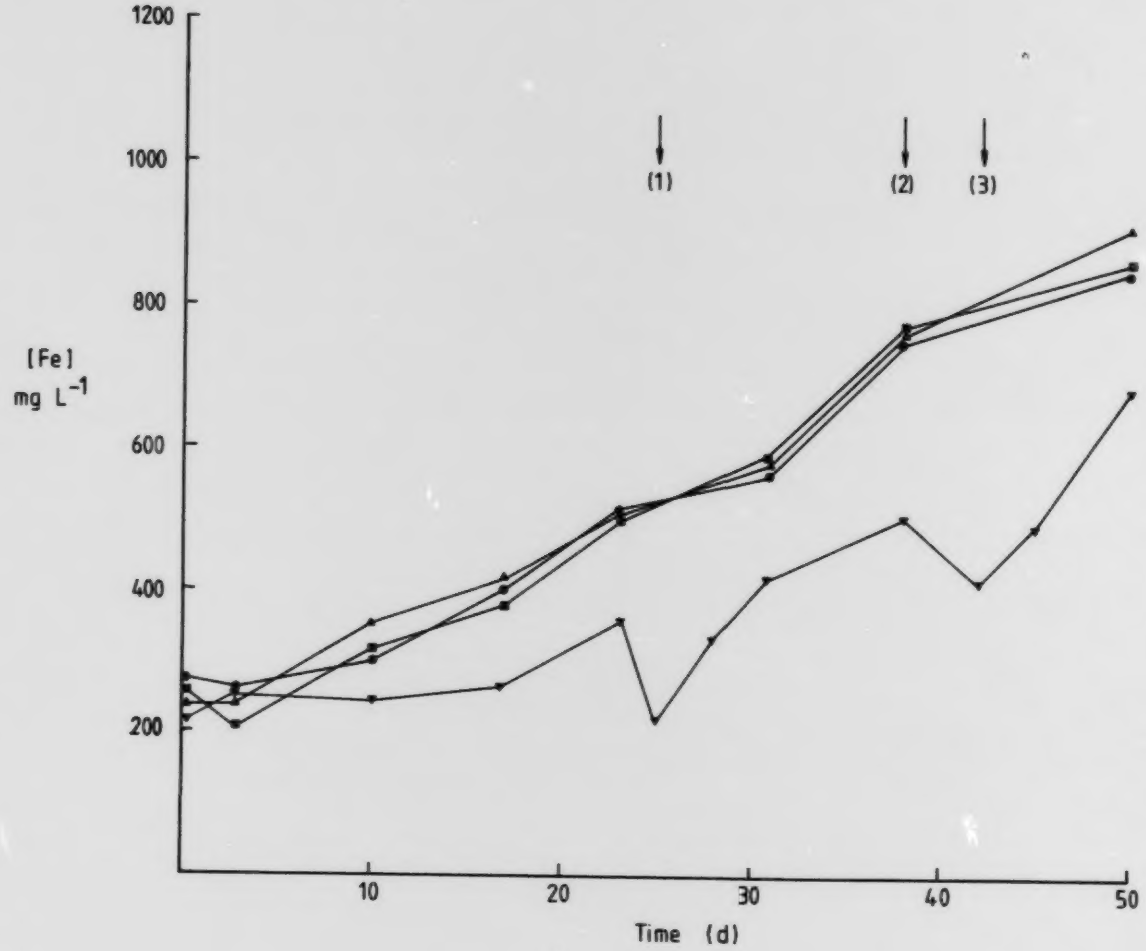

Fig. 2 Pyrite oxidation in pure and mixed cultures of *T. ferrooxidans* (NC1B 11820) in the presence of 10mM glucose: ● *T. ferrooxidans* (– glucose); ▲ *T. ferrooxidans* (+ glucose); ■ *T. ferrooxidans* + heterotroph SJH (+ glucose); ▼ *T. ferrooxidans* + heterotroph SJH (+ glucose). Arrows indicate amendments to ▼ culture: (1) and (2) glucose added at 10mM (final concentration); (3) basal salts added, as in original culture.

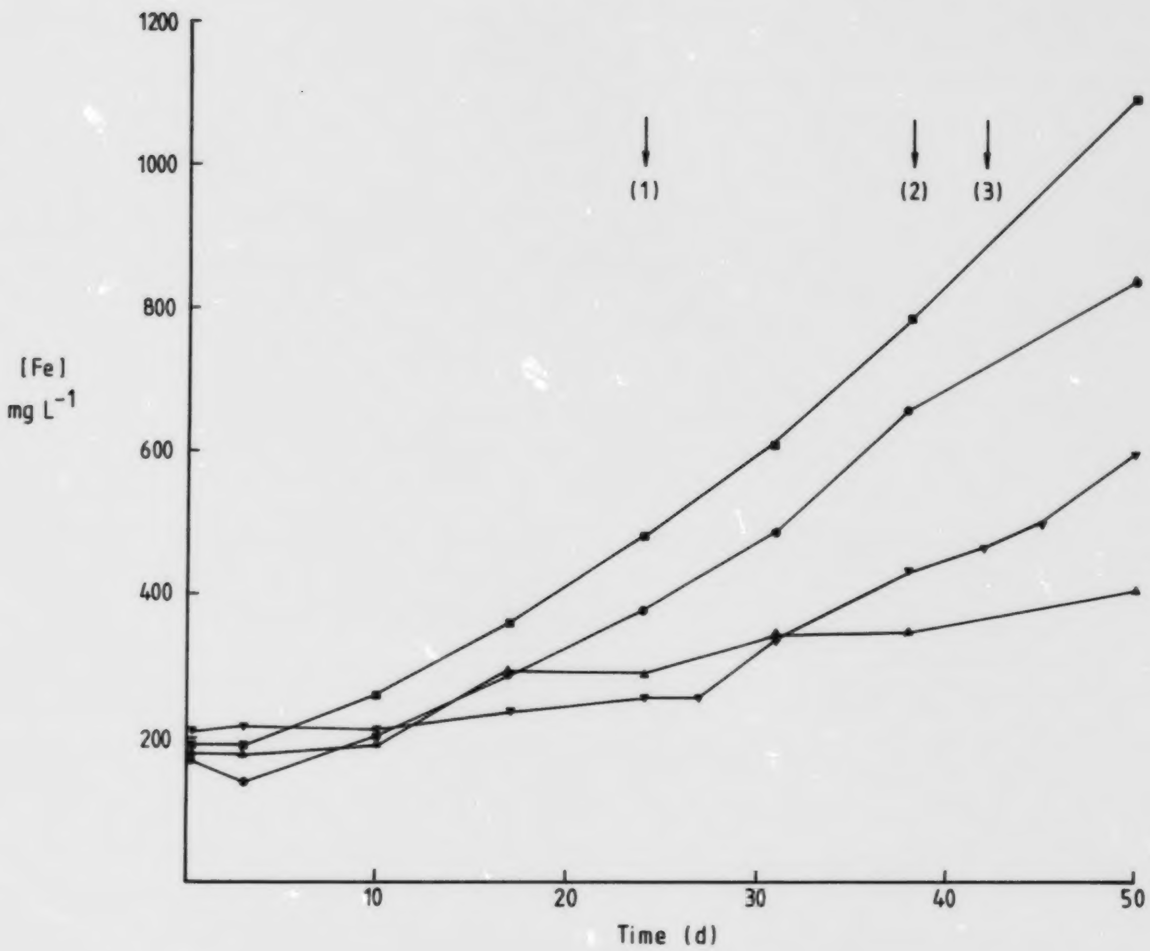

Fig. 3 Pyrite oxidation in pure and mixed cultures of *L. ferrooxidans* (isolate SJ1) in the presence and absence of 10mM glucose: ● *L. ferrooxidans* (-glucose);▲ *L. ferrooxidans (+glucose);* ■ *L. ferrooxidans + heterotroph SJH (-glucose).* ▼ *L. ferrooxidans + heterotroph SJH (+glucose). Arrows indicate amendments to* ▼ *culture: (1) and (2) glucose added at 10mM (final concentration); (3) basal salts added, as in original culture.*

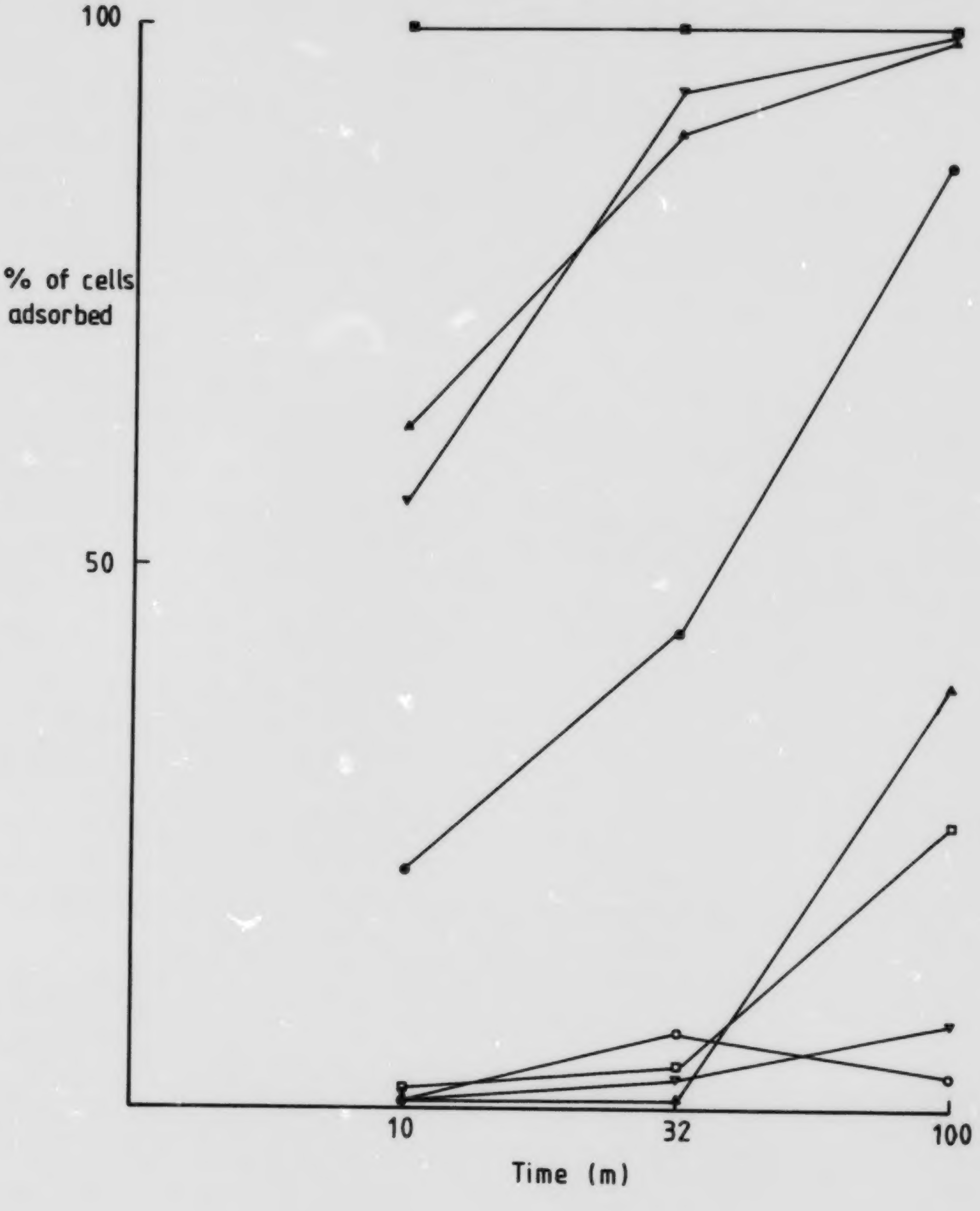

Fig. 4 Adsorption of some iron-oxidising bacteria onto pyrite (closed symbols) and glass (open symbols); ●, ○ *T. ferrooxidans* (NC1B 11820); ▲,△ *L. ferrooxidans* (isolate SY); ▼, ▽ *T. ferrooxidans* (isolate SJ2); ■, □ filamentous isolate OP14.

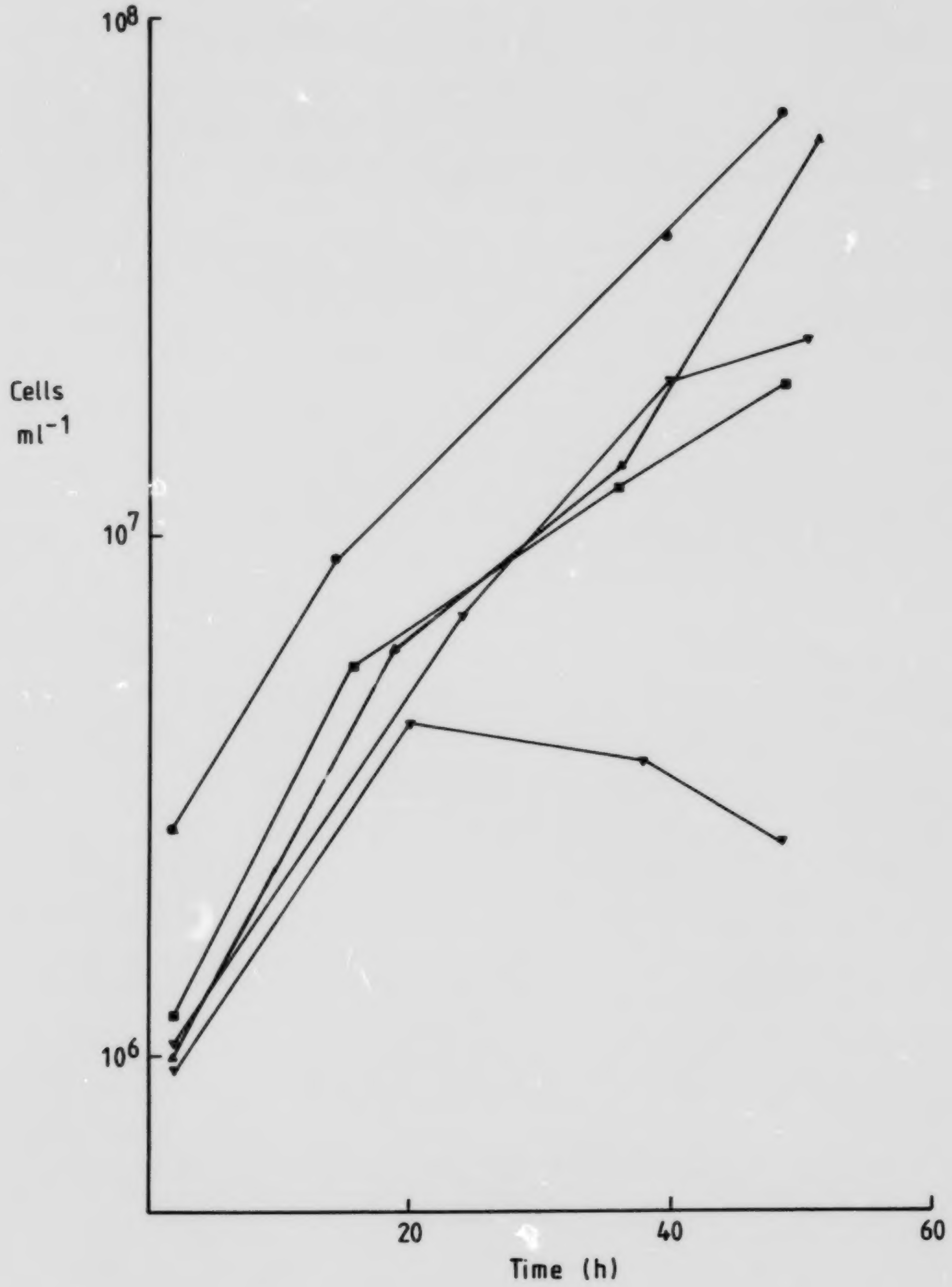

Fig. 5 Adsorption of some iron-oxidising bacteria onto ferric hydroxysulfate, based on numbers of unattached bacteria in pH 2.3 cultures: ● *T. ferrooxidans* (NC1B 11820); ▲ *L. ferrooxidans* (isolate SY); ▼ *T. ferrooxidans* (isolate SJ2); ■ filamentous isolate OP14; (▽ *T. ferrooxidans* SJ2, grown at pH 1.5 in the absence of a precipitate).

THE KINETICS OF SULFUR OXIDATION BY *Thiobacillus ferrooxidans*

*Miguel N. Herrera, Daphnne Alfaro, Blanca Escobar, Romilio T. Espejo, Tomás Vargas, Jacques V. Wiertz and Ricardo Badilla — Ohlbaum**

Department of Chemical Engineering, University of Chile, Casilla 2777, Santiago, Chile.

ABSTRACT

The kinetics of elemental sulfur oxidation by *T. ferrooxidans* were studied in a small flow leaching column packed with sulfur prills, employing a pure strain of *T. ferrooxidans* (ATCC 19859). For the direct oxidation of sulfur, a yield of about $1.2*10^7$ bacteria/μmol of sulfate produced and specific growth rate of 0.5 day^{-1} were determined in shake-flask experiments. Under continuous conditions, the bacterial yield is affected by the liquid flow rate, which affects the environmental conditions of the biooxidation process. Based on the experimental results, a generalized phenomenological model of the direct bioleaching process is presented. It incorporates the microbiological effect through a bacterial growth expression for the attached bacteria growing from direct oxidation of sulfur as well as the dependence of the growth with environmental changes. The model predicts well the measured pH, and the sulfate and bacterial concentrations in the effluent solutions under the different process conditions.

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* Corresponding author

LA CINÉTIQUE DE L'OXYDATION DU SOUFRE PAR *THIOBACILLUS FERROOXIDANS*

M. N. Herrera, D. Alfaro, B. Escobar, R. T. Espejo, T. Vargas, J. V. Wiertz et R. Badilla-Ohlbaum*

Département de Génie Chimique, Université du Chili, Santiago, Chili

RÉSUMÉ

Suivant un modèle composé de la bio-oxydation directe des minéraux sulfureux, la cinétique de l'oxydation du soufre élémentaire par *T. ferrooxidans* a été étudiée dans une petite colonne de lixiviation remplie de granules de soufre. Une souche pure de *T. ferrooxidans* (ATCC 19859) a été utilisée pour cette étude. Pour l'oxydation directe du soufre, un rendement d'environ 1,2 X 10^7 bactéries/μmol de sulfate produit et un taux de croissance spécifique de 0,5 jour^{-1} ont été mesurés dans des fioles. En continu, le rendement bactérien varie en fonction du débit du liquide, qui a une incidence sur le milieu dans lequel se produit la bio-oxydation. Un modèle phénoménologique généralisé du procédé d'oxydation bactérienne directe fondé sur les résultats d'expérience est présenté. Ce modèle fait état de l'incidence microbiologique en exprimant la croissance bactérienne à partir de l'oxydation directe du soufre ainsi que l'influence des variations du milieu sur la croissance. Le modèle donne une estimation adéquate du pH ainsi que des concentrations de sulfate et de bactéries dans les effluents sous diverses conditions.

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*Correspondant

INTRODUCTION

Thiobacillus ferrooxidans is considered to be one of the most important microorganisms involved in bacterial leaching processes. Bacteria affect the overall leaching rate by both indirect and direct biooxidation mechanisms. Indirect bacterial leaching involves the microbial oxidation of ferrous to ferric ions (by both free and attached populations) which are responsible for the chemical oxidation of sulfide minerals. Direct bioleaching proceeds through the interaction of bacteria with the surface of the sulfide minerals, sulfurs and other sulfur-containing compounds that are formed on the surface of the mineral particles during the process. This mechanism requires an contact of the cells with the mineral surface (see for example the reviews of Murr (1980) and Hutchins *et al* (1986)) such as *T. thiooxidans* attached to sulfur (Shaeffer *et al,* 1963) or pyrite (Rodriguez-Leiva and Tributsch, 1988), *Sulfolbus* attached to elemental sulfur (Weiss, 1973, Berry and Murr, 1975), and bacterial attachment to other sulfide minerals (Murr and Berry, 1976). Under practical bioleaching conditions, both types of mechanisms occur simultaneously, but their relative contributions to the overall leaching rate depend upon the characteristics of the reacting system and the ore (Herrera *et al, 1989).*

Espejo and Romero (1987) studied the growth of *T. ferrooxidans* using sulfur prills as the sole energy source. Their results indicated that the growth behaviour of the attached bacteria was exponential until saturation of the susceptible surface was reached, followed by a linear release of free bacteria due to succesive replication of the adsorbed bacteria. They also demonstrated that *T. ferrooxidans*, when ferrous iron is also present in the system, can simultaneously utilize both ferrous iron and sulfur as energy sources. Moreover, Espejo *et al,* (1988) showed that unadsorbed sulfur—grown cells oxidized ferrous iron at a rate 3 to 7 times slower than that of ferrous iron-grown *T. ferrooxidans* cells, but sulfur—grown cells were able to reach the oxidation rate of ferrous iron—adapted cells after only 1.5 generations in a medium containing ferrous iron, which clearly illustrates the constitutive nature of the iron and sulfur oxidation systems. They also reported that bacteria that are adsorbed onto sulfur prills oxidized ferrous iron at a rate similar to that of unadsorbed sulfur-grown bacteria.

Undoubtedly, bacterial attachment and direct bacterial oxidation play important roles in the bioleaching process but it is difficult to study direct oxidation separately from the indirect mechanism in the bioleaching of sulfide minerals, since under practical leaching conditions, the presence of iron interferes with both the kinetics and bacterial growth. Moreover, in industrial bioleaching operations, the process is carried out continuosly at conditions under which bacterial growth is limited by substrate and nutrients concentrations, which are far from those of shake-flask studies. Elemental sulfur is a good model compound to study the direct oxidation mechanisms in bioleaching, since no iron is either required or produced by the process. In this work the kinetics of sulfur oxidation by *T. ferrooxidans* were studied in small flow leaching column packed with sulfur prills, which simulates more closely the conditions of practical bioleaching processes and, in particular, bacterial growth under limiting oxygen supply. Based on the experimental results a model was developed which takes into account the more important aspects of the process.

MATERIALS AND METHODS

Sulfur prills. Spherical prills of elemental sulfur of a diameter between 2.85 and 3.33 mm were prepared following the method of quenching melted sulfur at 120°C described by Laishley *et al* (1986). The prills were sterilized together with the MS9B medium (Espejo and Romero, 1987) containing the following salts in grams per liter: $(NH_4)_2SO_4$, 0.1; $K_2HPO_4\cdot 2H_2O$, 0.04; $MgSO_4\cdot 7H_2O$, 0.25 and $FeSO_4\cdot 7H_2O$, 0.03 in an autoclave at 100°C for 30 minutes on 2 successive days.

Bacterial Strain. Thiobacillus ferrooxidans ATCC 19859 was used in all experiments. The strain was previously adapted to grow in the MS9B medium by at least five transfers.

Bacterial Activity. The microbial growth kinetics of the *T. ferrooxidans* strain employed in the column experiments were evaluated first in shake-flask experiments. The reaction was followed by counting of free bacteria and analysis of sulfate ions in the solution. The determination of bacterial numbers was performed as described elsewhere (Espejo and Romero, 1987). The amount of sulfate produced was measured by a turbidometric procedure based on excess barium chloride precipitation. From the experimental data, a value of $1.2 \cdot 10^7$ bacteria/μmolSO4^{-2} for the bacterial yield (Y) was obtained. The specific growth rate (μ), obtained from the slope of the curve showing bacteria numbers in solution vs time, was found to be 0.5 day-1. The value for the maximum number of adsorbed cells on the surface (N_s) was obtained from the equation (Espejo and Romero, 1987):

$$\mu = \frac{\Delta N_f}{\Delta t * N_s} \tag{1}$$

where N^f is the number of free bacteria in solution. Equation 1 implies that the specific growth rate does not vary significantly once the available surface of the prills is saturated. The value of the maximum number of cells that can be attached onto the external surface of the sulfur prills is about $1.03 \cdot 10^8$ bact/cm^2 under the conditions employed.

Experimental Apparatus and Procedure.

A schematic diagram of the experimental apparatus employed in the present work is given in Figure 1. The flooded column (a) was a 5 cm height and 1.7 cm diameter glass pipe inmersed in a temperature controlled bath (p) at 30°C (o) and loaded with about 500 sulfur prills. A controlled flow of the basal medium MS9B from a 12 L feeding tank (n) was fed continuously (c) by means of a Cole Palmer 7554 peristaltic pump (l) to a 1.2 L agitated sealed tank (b,f,g,h) to which a controlled flow (k,j) of filtered air(e) saturated in water (i) was bubbled (d) in order to saturate the inlet solution with oxygen. The experiments were monitored by analysis of the effluent solutions for free bacteria counts and pH. The inlet pH of the solution was adjusted at 5.0 and the feed flow was varied in a range of 0.12 to 11.2 ml/min. For the studies of the effect of the inlet pH on the growth of *T. ferrooxidans*, a constant feed flow value of about 0.057 ml/min was adjusted and the pH was varied in a range of 2.55 to 5.43.

RESULTS AND DISCUSSION

Experimental results

The experimental results of the effect of the pH of the inlet solution on the free bacteria counts in the effluent solutions are shown in Figure 2. The results indicate that bacterial counts increase as the pH of the inlet solution increases, reaching a maximum at about pH 4 to 5. Therefore, *T. ferrooxidans* cells grow better with sulfur at a pH between 4 and 5, which is somewhat higher than the optimum value for growing with ferrous iron (Ingledew, 1982), in agreement with the early results of Silver (1978). Figure 3(a,b) shows the experimental results and model predictions of the effect of the feed flow rate (at pH 5) on the numbers of free bacteria and the sulfate concentration measured in the effluent column solutions. For both numbers free bacteria and sulfate ion concentration, a linear behaviour is observed when the reciprocal values of the formed SO4^{-2} ions and Nf are plotted (Figure 3a and 3b) at flow rates above 2.0 ml/min. This observation indicates that nutrient concentrations are not rate-limiting at these flow-rate ranges and the oxygen supply is not limiting for the oxidative metabolic system of *Thiobacillus ferrooxidans* growing on elemental sulfur. However, at flow rates below 2.0 ml/min, the rate of oxygen supply is limiting, which in turn affects the rate of bacterial growth and hence the rate of sulfur oxidation. In fact, at a flow rate of 2 ml/min, the total supply of oxygen into the column is 0.5 μmol/min whereas, since the minimum oxygen requirement of the system for both bacteria and the stoichiometry of the sulfur oxidation reaction is about $4.34 \times 10^{-1}1$ μmol/bact•min (Silver (1978) reported a value of $1.0 \times 10^{-1}1$ μmol/bact•min for the metabolic oxygen consumption by *T. ferrooxidans* , the total oxygen requirement of the the column is about 0.62 μmol/min. This value is close to the total supply of oxygen, thus corroborating the experimental finding that at flow rates below 2.0 ml/min, oxygen is indeed a limiting factor for the biooxidation process.

Figure 4 shows the effect of the liquid flow rate on the bacterial yield, calculated from the values of the free bacteria counts and sulfate concentrations in the effluent solutions. The results suggest that the bacterial growth is not directly proportional to the amount of sulfur oxidized when the feed flow rate is varied under continuous cultivation conditions.

Model Formulation

In the present work, the column contained only sulfur prills that were metabolically oxidized by adsorbed *Thiobacillus ferrooxidans* cells according to

$$2S^o + 3O_2 + 2H_2O \xrightarrow{T.ferrooxidans} 2SO4^{-2}\ 4H^+ \qquad (2)$$

Thus, the elemental sulfur oxidation rate can be described in terms of the rate of bacterial growth and the bacteria yield as:

$$\frac{dS^o}{dt} = \frac{1}{Y} * \frac{dN_f}{dt} \qquad (3)$$

whereas the growth of free bacteria in the solution (N_f) can be related in terms of the number of attached cells (N_s) and the specific growth rate (μ) according to Espejo and Romero (1987) by:

$$\frac{dN_f}{dt} = \mu * N_s \qquad (4)$$

Pirt (1965) showed that for bacterial growth under continuous cultivation conditions, the yield is related to the growth (Y_{max}) and a maintenance coefficient (m_s) that becomes more important at low dilution rates. Although in the present work bacteria are growing attached to the surface of the prills and therefore are not directly affected by the flow rate, the same approach can be employed to take into account the experimental fact that the calculated yield values at different flow rates (from the results presented in Figure 3) are not independent of the measured flow rate, so that:

$$\frac{1}{Y} = \frac{1}{Y_{max}} + \frac{m_s}{\mu} \qquad (5)$$

and the maintenance coefficient is related to the flow-rate (F) by:

$$m_s = \frac{\alpha}{F} \qquad (6)$$

in which α is an experimental constant. From the data presented in the Figure 4, the values of $4.68x10^6$ bact/μmol $SO4^{-2}$, 5.98x10-8 μmolSO4^{-2}/bact•ml and 8.33x10-3 min-1 for Y_{max}, $\alpha$and μwere obtained by a linear regression analysis of the coefficients of Equations 5 and 6.

Equations 2 to 6 give the mathematical description of the bacterial sulfur oxidation process that occurs in the column. For the column, the mass balance expression for each species i present in the liquid in the axial direction of the column is:

$$\varepsilon L \frac{dC_i}{dt} + v_z \frac{dC_i}{dz} * \frac{d}{dz}\left[D_{ai} \frac{dC_i}{dz}\right] = R_i \qquad (7)$$

where ε L is the volume fraction of liquid per unit volume of packed bed, C_i is the concentration of species i in the solution, vz is the superficial liquid velocity and D_{ai} is the axial dispersion coefficient of specie i. R_i represent the rate of change in the concentration due to the chemical reaction and/or transport of the species i from or into another phase per unit volume of packing. In Equation 7, the first term on the left hand side represents the accumulation, the second term the changes in concentration within the column, and the third term, the changes in the column due to non-ideal flow patterns. Due to the inherent slow kinetics of the sulfur oxidation process, pseudo-steady state conditions can be assumed and the liquid- solid mass transfer resistances can be neglected. Considering the characteristics of the small reactor employed in the present work, a uniform flow distribution can be also assumed. Therefore, Equation 7 can be reduced to:

$$v_z \frac{dC_i}{dz} = R_i \qquad (8)$$

The resulting set of coupled ordinary differential equations that follows from the model described above was solved numerically using the 4th order Runghe-Kutta integration algorithm.

Model Application

Comparisons of the experimental results and the model prediction for free bacteria and sulfate ion concentrations in the effluent solution are shown in Figure 3. The results indicate that free bacteria counts and sulfate concentrations in the effluent solution increase as the feed flow rate decreases. Figures 5 and 6 shows model predictions for dissolved oxygen and pH profiles inside the column at feed flow rates of 2.0, 3.4, 6.77 and 11.2 ml/min.

From the results presented in Figure 3, the values of the bacterial yield, i.e. the ratio between free bacteria counts and the mass of sulfur oxidized, can be calculated at the studied flow rates, and they show that the yield increases with the flow rate (between 1.1×10^6 to 4.68×10^6 bact/μmol $SO4^{-2}$ when the flow rate increases from 2 to 11.2 ml/min, Figure 4). These values are lower than those determined in the shake-flask experiments (about 1.2×10^7 bact/μmol $SO4^{-2}$) which clearly indicates that under continuous flow, the presence of environmental stress such as the dissolved oxygen concentration and pH changes throughout the column (shown in Figures 5 and 6) affect the growth mechanism of the bacteria (Jerez *et al*, 1988), suggesting that a fraction of the energy from the oxidized sulfur is employed for endogeneous metabolism. This effect is more important as the flow rate decreases and, as in the present case, becomes critical as the flow rate reaches a value of about 2 ml/min, when dissolved oxygen is depleted in the first 4 cm of the column (Figure 5d).

These findings are important considerations for the understanding of bioleaching industrial operations since the attached bacterial population plays a crucial role in the process. Moreover, they clearly illustrate the role of dissolved oxygen concentrations on the kinetics of the biooxidation process.

CONCLUSIONS

A study of the kinetics of direct oxidation of elemental sulfur by *T. ferrooxidans* was carried out in a laboratory-scale leaching column which simulates, at laboratory scale, process conditions similar to those of industrial bioleaching processes. Bacterial action can be correlated with direct sulfur oxidation, and the rate of the process is affected by the concentration of dissolved oxygen in the column, which in turn, is determined by the liquid flow rate employed in the reactor. Bacterial yield under continuous flow conditions is lower than that when the oxidative process is carried out in shake-flask conditions and is affected by the flow rate, which is an indication that the growth of *T. ferrooxidans* associated with the direct bioleaching mechanism is affected by the environmental conditions.

A model to describe elemental sulfur oxidation by *Thiobacillus ferrooxidans* has been presented, which is capable of predicting well the experimental results and gives a practical tool for management of industrial bioleaching operations.

ACKNOWLEDGMENTS

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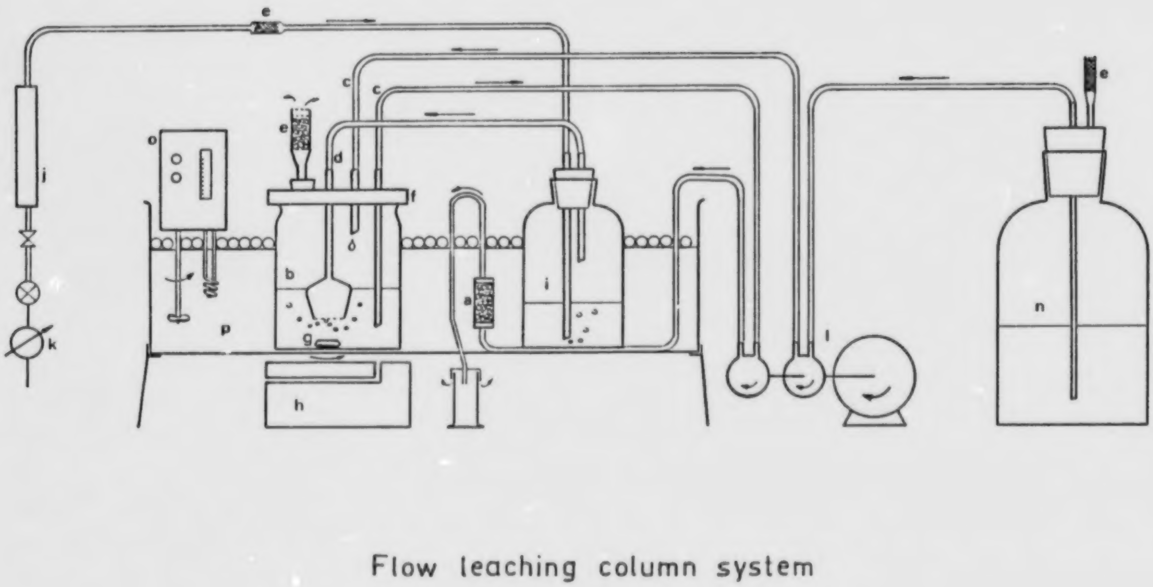
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Flow leaching column system

Fig. 1 Experimental flow leaching column system.

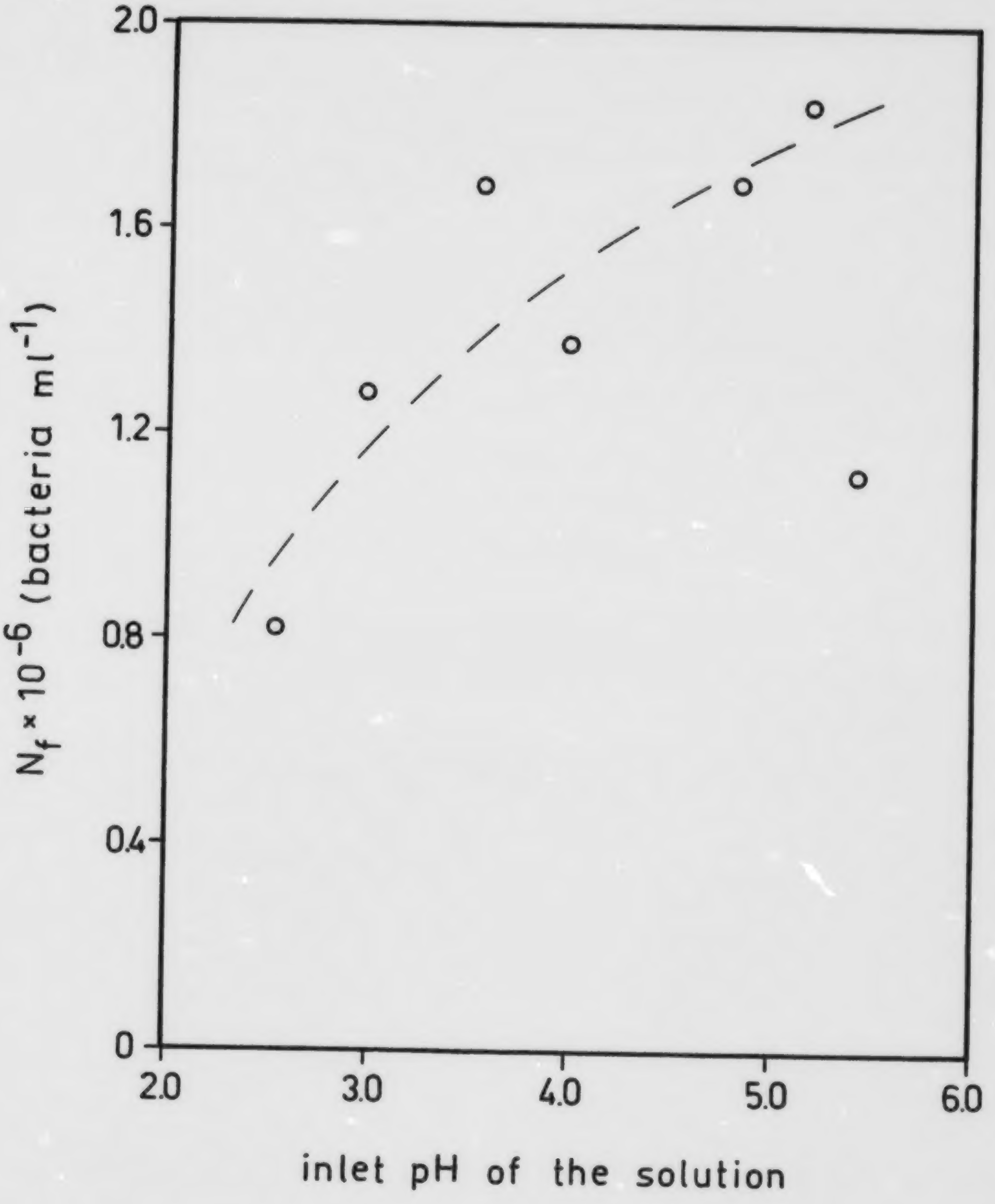

Fig. 2 Effect of the pH of the feed on the numbers of bacteria and in the column effluent solutions.

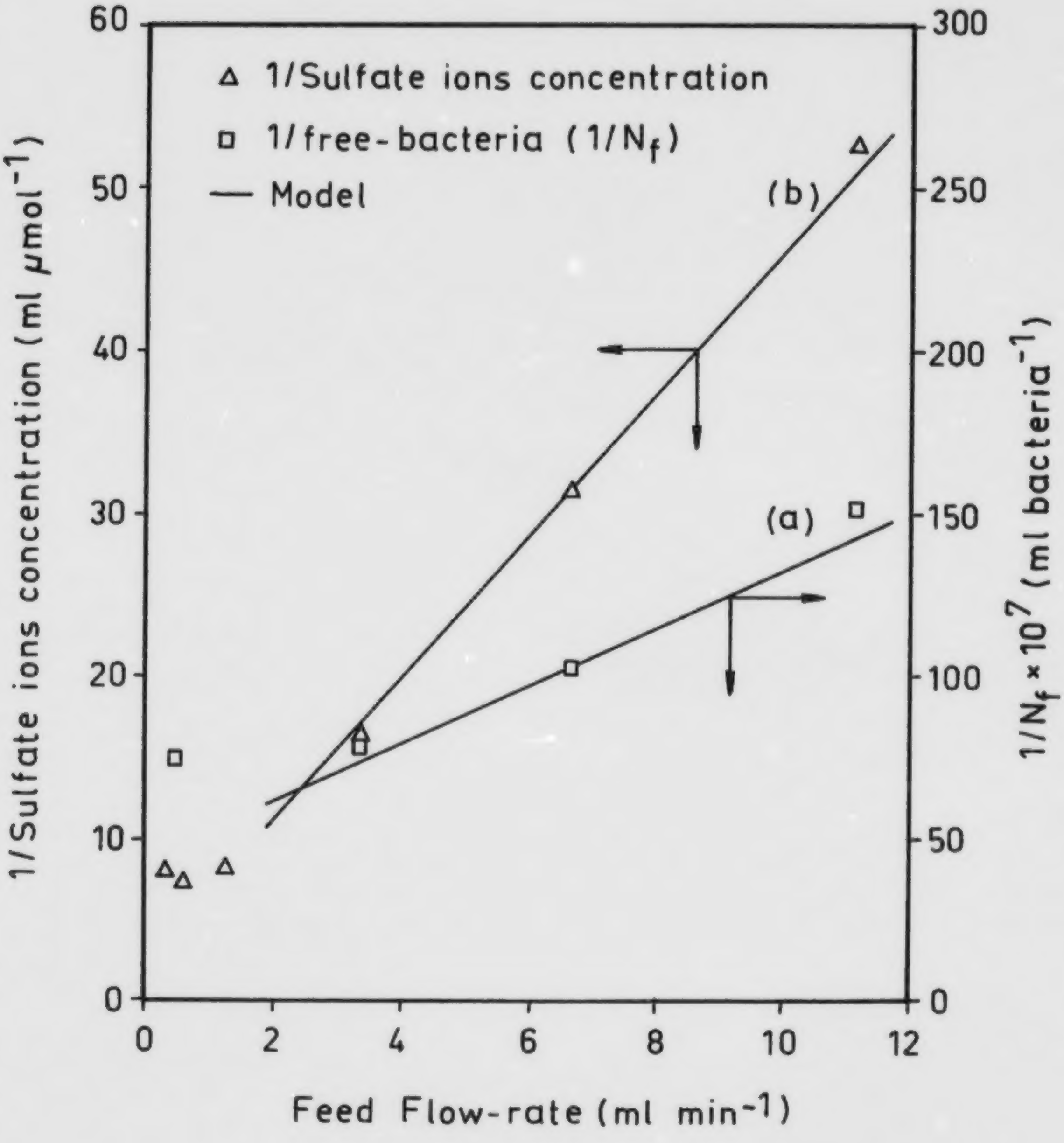

Fig. 3 Effect of the feed flow-rate on the numbers of bacteria and sulfate concentration in the outlet solution. Curves (a) experimental points and model prediction for sulfate ions concentrations; (b) experimental points and model predictions for free-bacteria counts.

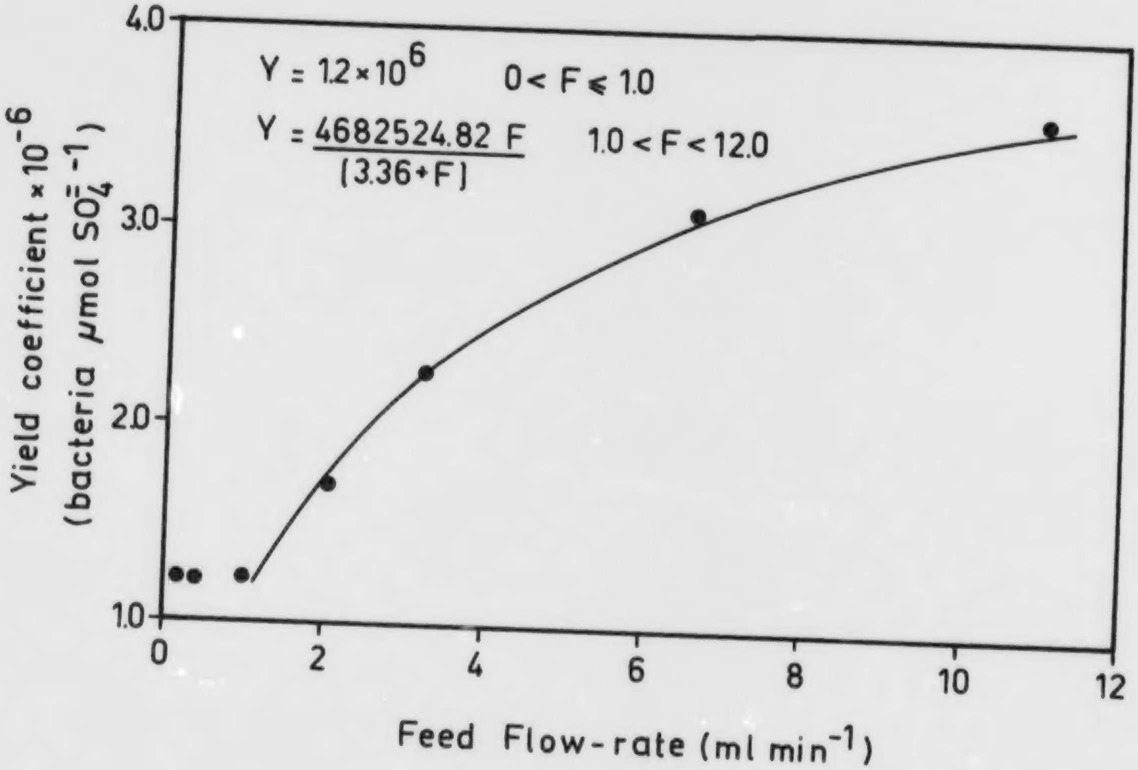

Fig. 4 Effect of the liquid flow rate on the bacterial yield.

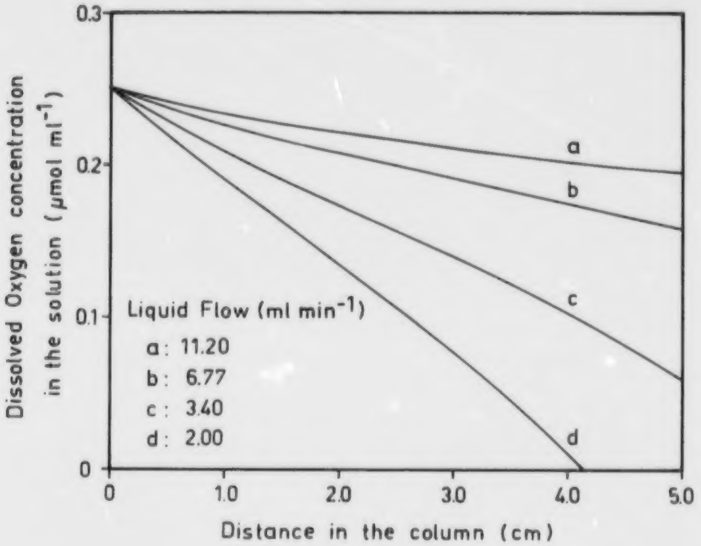

Fig. 5 Effect of the feed flow-rate on the axial profiles of dissolved oxygen concentrations in the column.

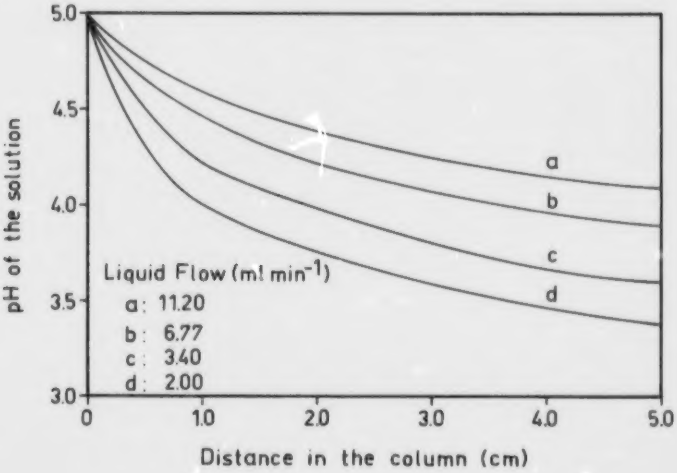

Fig. 6 Effect of the feed flow-rate on the pH profiles in the column.

INVESTIGATIONS ON LEACHING BACTERIA BY MICROCALORIMETRY

Schroeter, A.W.; Sand, W.

University of Hamburg, Institute for General Botany, Department of Microbiology
Ohnhorststrasse 18, 2000 Hamburg 52, FRG

Summary

A new technique microcalorimetry for the characterization of chemolithotrophic bacteria and the evaluation of their importance in leaching processes has been developed. Experiments under isothermal conditions with pure cultures revealed that species and different strains of species of thiobacilli, leptospirilli, and nitrifying bacteria can be differentiated by their power-time curves. The investigation of nitrifying bacteria was included because they were found to be present in acidic and alkaline mine samples. Their metabolic endproduct, nitric acid, yields even at neutral pH-values soluble metal salts. The differences in the power-time curves are strain-specific and may be used for the characterization of unknown pure cultures. The comparison of the power-time curves of defined mixed cultures with those of pure cultures demonstrated that the power-time curve was an addition of the single curves and that predominant organisms in mixed populations may be addressed. The integral of the heat-output (power x time, energy) is a measure of metabolic activity, and may be used for a calculation of the efficiency. Good growth is correlated with low power generation. The perfusion vessel, a mini-reactor of 3ml volume, allows experiments with solid materials under constant control on line. It may be used for a detection of relevant organisms, their characterization, and the estimation of their leaching abilities.

ÉTUDES DE BACTÉRIES RESPONSABLES DE LA LIXIVIATION PAR MICROCALORIMÉTRIE

Schroeter, A.W. et Sand, W.

Université de Hambourg, Institut de Botanique Générale
Département de Microbiologie Hambourg, R.F.A.

RÉSUMÉ

Une nouvelle technique, la microcalorimétrie, a été mise au point pour permettre la caractérisation des bactéries chimiolithotrophes et l'évaluation de leur importance dans le cadre des procédés de lixiviation. Des expériences faites en conditions isothermes et sur des cultures pures ont révélé que des espèces et différentes souches de *Thiobacillus* et de *Leptospirillum* ainsi que des nitrobactéries peuvent être identifiées à partir de leur courbe de dégagement de chaleur. L'étude des nitrobactéries a été incluse parce que ces bactéries sont présentes dans les échantillons de minerais acides et alcalins. L'acide nitrique produit par leur métabolisme donne, même en milieu à pH neutre, des sels métalliques solubles. Chaque souche produit une courbe de dégagement de chaleur particulière, de sorte que ces courbes peuvent permettre de caractériser des cultures pures inconnues. La comparaison entre les courbes obtenues à partir de cultures mélangées définies et celles provenant de cultures pures ont permis de déterminer que la courbe obtenue correspond à l'addition des courbes propres à chaque bactérie présente. Ainsi, dans le cas de populations mixtes, il est possible d'isoler l'élément prédominant. L'intégrale du dégagement de chaleur (puissance x temps, énergie) est une mesure de l'activité métabolique et elle peut servir à calculer l'efficacité.

Si la croissance est bonne, le dégagement de chaleur est faible. Le récipient de perfusion, miniréacteur de 3 mL, permet de procéder à des expériences sur des solides sous contrôle constant. Ce miniréacteur peut servir à déceler des organismes pertinents, à les caractériser et à évaluer leurs qualités en matière de lixiviation.

Introduction

For research work on the degradability of ore and industrial residues by chemolithotrophic bacteria microcalorimetry was chosen, because this technique allows the control of the degradation of substrates due to its measuring principle on-line (Spinck and Wadsö, 1976). The heat, which is released from biochemical redox-reactions, may be used as a direct measure for microbial activity. The applicability of heat-flow measurements was tested with pure cultures and soluble substrates like ferrous iron, thiosulphate (Schröter and Sand 1988), ammonia, and nitrous acid in a flow-through mode under variation of temperature, aeration, flow-speed, and inoculum. Some experiments were made with the thermophilic sulfur-oxidizing *Sulfolbus brierleyi* at 65°C in closed, 4,5ml containing ampoules with sulfur as substrate (Schröter and Sand, 1988) and also with the moderately thermophilic *Thiobacillus tepidarius* at 44°C with thiosulfate as substrate. These tests resulted in an evaluation of the optimum conditions for the experiments with relevant leaching organisms.

The perfusion vessel, a type of bioreactor with 3ml volume, which may be aerated and stirred, is used for the tests on the degradability of ore and industrial residues. With the first experiments on soluble substrates the basic features of this method were tested. Later on substrates like pyrite and ore were used in experiments with thiobacilli and leptospirillum-like organisms.

For an evaluation of leaching capabilities of nitrifying bacteria growth experiments with ammonia as well as nitrous acid oxidizing bacteria were carried out.

Material and Methods

A Thermal Activity Monitor (TAM, Thermometric AB, Bromma, Sweden or C-3-Analysentechnik GmbH, Vaterstetten, FRG) was used for the isothermal heat conduction measurements (Nordmark *et al.*, 1984; Sand, 1987). The flow-through experiments were conducted at 30°C in combination cylinders. The flow-rate amounted to 20ml/h culture-suspension and 20ml/h air, which were pumped alternatively for aerobic conditions. The experimental set up is given in Fig.1.

For a constant pH-value in the bacterial culture an automatic titraton was installed.

The organisms were cultured according to Milde *et al.* (1983, *T. intermedius*), Mackintosh (1978, *T. ferrooxidans*, *L. ferrooxidans*), and Wood and Kelly (1977, T. versutus).

The perfusion-vessel was used with the titration shaft connected with a cylindric turbine stirrer and additionally inserted canulae for aeration (Fig.2).

The perfusion-experiments were made at 30°C, the liquid volume amounted to 2ml, the stirrer was run clockwise in a discontinuous mode (5s-cycle) at 120rpm, and the aeration rate with prewarmed, humidified air totalled 2,7ml/h.

The bacterial cells were harvested at 27000 x g, washed with sterile basal salts solution, and sedimented again (50000 x g). The cell numbers were adjusted to $1x10^{10}$ cells/ml ($\pm 5 \times 10^9$).

As solid substrates, pyrite (20-50μm grain size) and a sulfidic ore (0-50μm grain size) from Ilba Venera (Romania) were used. The analytical data are given in Tab.1.

Results and Discussion

Flow-through-experiments

(1) Thiosulfate oxidation with and without constant pH-value.If thermograms of different strains were compared, differences in heat-output were noted (Schröter and Sand, 1988). Good growing strains

produced less power than poorly growing ones (results not shown). As this might be due to a change in pH-value during substrate oxidation, the influence of the pH-value on the heat-output was tested. Thus, with a titration-unit the pH-value was kept constant at the optimum value of each strain. As an example Fig.3 shows the power released by *T. versutus* strain DSM 582 during autotrophic thiosulfate-oxidation without pH-correction (left) and with pH-correction (right). This sulfuric acid producing bacterium has a pH-optimum between 7.6 and 7.9. The thermograms show that at pH-values below optimum an increased heat-output results. The growth efficiency is reduced. The generation-time increased under suboptimal conditions from 15h to 29h.

(2) Influence of oxygen on thiosulfate oxidation. Most autotrophic bacteria are able to grow even at low oxygen partial pressure (pO_2) (Myerson, 1981). However, many thiobacilli grow best at high oxygen saturation levels. Fig.4 shows the results of experiments with *T. intermedius* D 14 at different oxygen saturation levels. It is obvious that a reduced oxygen supply results in prolonged intervals between the peaks. The cells regulate their metabolism accordingly.

(3) Ferrous iron oxidation with *T. ferrooxidans* (Tf) and *L. ferrooxidans* (Lf). Iron oxidizing bacteria were isolated from sulfidic Romanian mines. Their growth patterns are shown in Fig.5. Concerning the heat-flow characteristics, the *Tf*-isolate is quite different from well known *Tf*-strains, while the *Lf*-strain shows little differences to the previously tested strains (Schröter and Sand, 1989). The results confirm that the "species" *T. ferrooxidans* is per definitionem only a phenospecies and consists of genetically heterogenous members (Harrison, 1982).

(4) Pure and mixed cultures of iron oxidizing bacteria. For an evaluation of a natural or an artificial habitat information about the composition of the population are needed. Microcalorimetry may be used to determine rapidly the potential for biological degradation and the predominant organism. Experiments with defined pure and mixed cultures were carried out, which showed some common features. From two stock-cultures of *T. ferrooxidans* ATCC 19859 and *L. ferrooxidans* AS1 one mixed culture and two pure cultures were prepared and inoculated into ferrous iron solution. The tests were started with identical cell numbers and identical growth conditions. The thermogram of the mixed culture exhibited characteristics of those of the two pure cultures. Microscopic observations, which were done continuously during the experiment, revealed a successive growth of firstly rods and secondly spirilla. The results are shown in Fig.6 together with those of the two pure cultures. The successive growth is in accordance with data for generation times, which give values for *T. ferrooxidans* of 8h and for *L. ferrooxidans* of 25h.

(5) Nitrous acid producing bacteria — Ammonia oxidation by *Nitrosovibrio spec.*. Ammonia oxidizing nitrifiers of a newly isolated species of Nitrosovibrio, whose metabolic end-product nitrous acid is accumulated in the medium, have been investigated during growth in a buffered batch culture. This species was tested, because it was shown to be the most abundant ammonium oxidizing bacterium in decaying sandstone, and thus, is actively involved in the degradation of natural stones (Meincke *et al.*, 1989, Bock and Krumbein, 1989). The thermogram (Fig.7) displays three main heat producing events, which can be caused by enzyme induction due to a stepwise substrate oxidation, or changes in growth conditions (pH- or pO_2-values), or end-product inhibition by nitrite. Further work is needed for an interpretation of these results. Nevertheless, it was shown that even this very slow growing organism with its little energy providing substrate can be investigated by this technique.

(6) Nitric acid producing bacteria — nitrous acid oxidation by *Nitrobacter winogradskyi*. Fig.8 shows a thermogram of *Nitrobacter winogradskyi* ATCC 255 during lithoautotrophic growth with nitrous acid (7,5mMol) and aerobic conditions. The single-peak thermogram suggests that nitrous acid is oxidized in an one-step reaction to nitric acid, which is excreted into the nutrient solution. The enzyme system is constitutive, as has been described by Sundermeyer and Bock (1981). This fact is reflected by the power-time curve. The induction of the key enzyme nitrite-oxidoreductase is completed after 15h. Nitrite is already oxidized at a linear rate. The induction is followed by a strong increase of the heat-output. At depletion the output drops sharply.

Perfusion-experiments:

(1) Oxidation of ferrous iron. The perfusion vessel allows experiments with solid substrates under aerobic conditions in a limited volume. Thus small amounts of sample and comparably little inocula are needed. The registration of the metabolic activity is achieved by the on-line measurement of the heat-output.

The first set of experiments was carried out with ferrous iron. The soluble substrate was chosen because many results of microcalorimetric measurements were available. They are not fully comparable due to the measuring mode, which usually was of the flow-through type. Fig. 9 gives the results of three micro-calorimetric experiments. Line "a" represents an experiment with 180mMol ferrous iron under sterile conditions. Line "b" of Fig. 9 displays the second control experiment, where 10^{10} cells/ml of *T. ferrooxidans* ATCC 19859 without substrate in the basal salts solution were monitored. This line gives the heat-output of endogenous metabolism. Finally, line c indicates clearly the metabolic iron oxidation activity of *T. ferrooxidans* ATCC 19859 (10^{10} cells/ml). Thus, the perfusion-vessel maybe used for studies on metabolism of *T. ferrooxidans*.

(2) Oxidation of pyrite and ore. Having demonstrated that the perfusion-vessel maybe used with *T. ferrooxidans*, experiments with solid substrates were designed. As has been mentioned, they aimed at the development of a method for the rapid detection of the microbiological degradability of unknown substrates. In analogy to the experiments with soluble substrates several experiments were necessary. The first microcalorimetric experiment with 43mg pyrite in 2ml basal salts solution was done under sterile conditions. The on-line measurement of the heat-output is given by curve "a" in Fig. 10. The experiments with *T. ferrooxidans* strain R7 (isolate from Romania) and strain ATCC 19859 are also included in Fig. 10. The graphs b and d represent the results. Strain R7 shows clearly less pyrite oxidation activity than strain ATCC 19859. The latter strain was tested in the fourth experiment, due to its high pyrite oxidizing activity, for its ability to attack a sulfidic ore from Romania. Initially the rise in heat-output, which is demonstrated by curve c in Fig. 10, is comparable to the experiment with pure pyrite. But soon a decrease is noted. Nevertheless, till the end of the experiment substantial heat-output was measured. As the ore consists only to several percent of degradable sulfides, it is consistent that in comparison to pure pyrite the metabolic activity of the bacteria decreases.

Chemical analysis of the content of the vessel showed in each case that under sterile conditions ferrous iron was released, whereas in the inoculated samples only ferric iron could be measured (results not shown).

Conclusions

The results of this work show that the technique microcalorimetry may be applied to basic research with lithotrophic bacteria as well as to problems of the leaching process. It could be shown directly that suboptimal growth conditions result in reduced metabolic efficiency of the bacteria. This fact may be used for process control. Another important result is the detection of predominant organisms in mixed cultures. Compared to conventional methods, microcalorimetry allows within short time, an evaluation of the important bacteria. In addition the work with the perfusion vessel shows, that this technique may be used for an evaluation of a microbiological degradability of ore.

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Table 1

Analysis of the solid substrates for the perfusion experiments:

1. Pyrite flotation concentrate (supplied by Scanore, Hamburg, FRG)

compound	Cd	Cu	Ni	Co	Pb	Sb	Zn	As
(mg/kg)	8	310	20	180	380	100	1600	41

2. Sulfidic ore from Ilba Venera (Romania):

compound	SiO_2	S	Fe	Cu	Al	Mn	Mg	Ca
(g/kg)	500	70	46	2	31	5	17	3
compound	Zn	Ag	Ga	V	As	Pb		
(mg/kg)	1200	12	10	20	750	400		

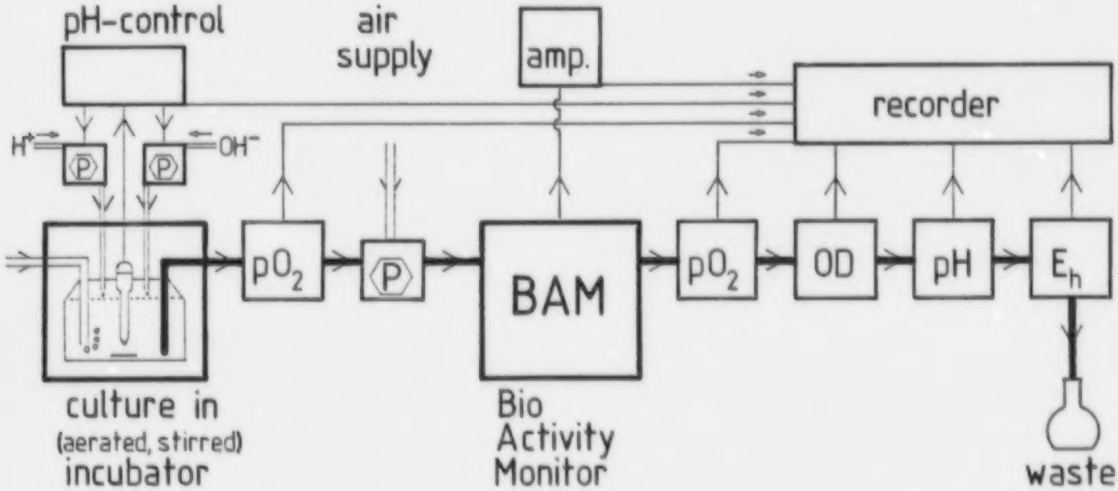
pH-control
air supply
amp.
recorder
H⁺
OH⁻
P
pO2
P
BAM
pO2
OD
pH
Eh
culture in (aerated, stirred) incubator
Bio Activity Monitor
waste

Fig. 1 Experimental setup for microcalorimetric aerobic flow-through measurements with additional installations.

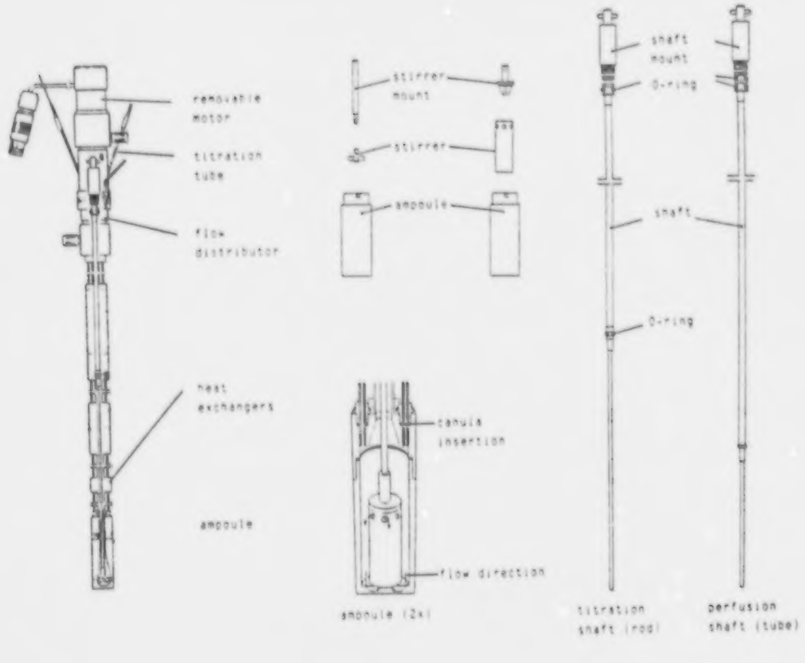
removable motor
titration tube
flow distributor
heat exchangers
ampoule
stirrer mount
stirrer
ampoule
canula insertion
flow direction
ampoule (2x)
shaft mount
O-ring
shaft
O-ring
titration shaft (rod)
perfusion shaft (tube)

Fig. 2 Diagram of the perfusion vessel for growth experiments with solid substrates.

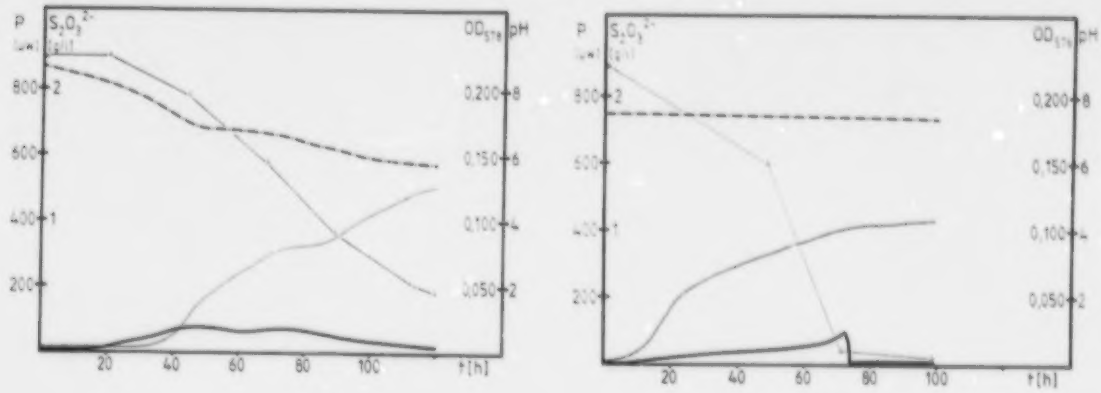

Fig. 3 Thermograms of T. versutus DSM 582 oxidizing 20 mMol thiosulfate without (left) and with (right) pH-correction. Flow-through mode including aeration (2x20ml/h), 30°C.

P (μW) = ▬▬▬▬▬ $S_2O_3^{2-}$ (mg/l) = Δ ——— Δ
pH = ▬ ▪ ▬ ▪ ▬ OD_{578} = - - - - - - -

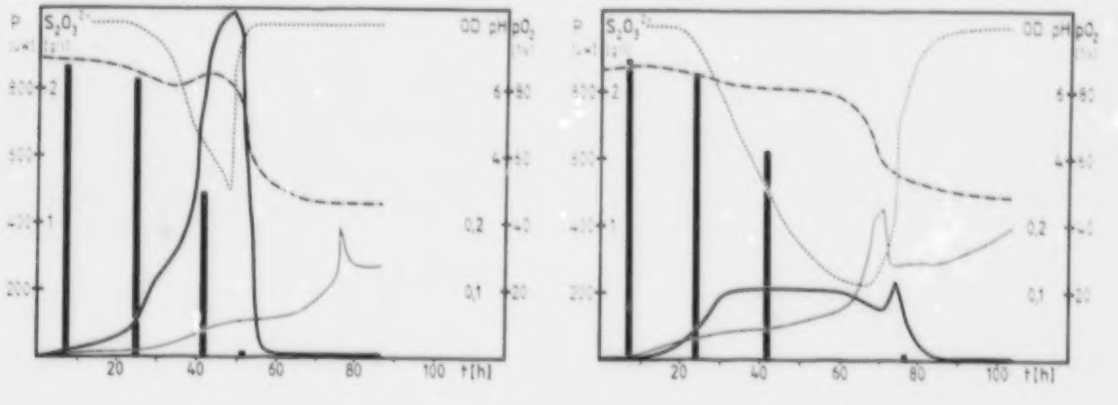

Fig. 4 Thermograms of T. intermedius D 14 grown autotrophically with 20mMol thiosulfate under high (left) and reduced (right) oxygen saturation. Flow-through mode.

P (μW) = ▬▬▬▬▬ $S_2O_3^{2-}$ (mg/l) = ■
pH = ▬ ▪ ▬ ▪ ▬ OD_{578} = - - - - - - -
pO_2 (%) = ▰▰▰▰▰▰▰

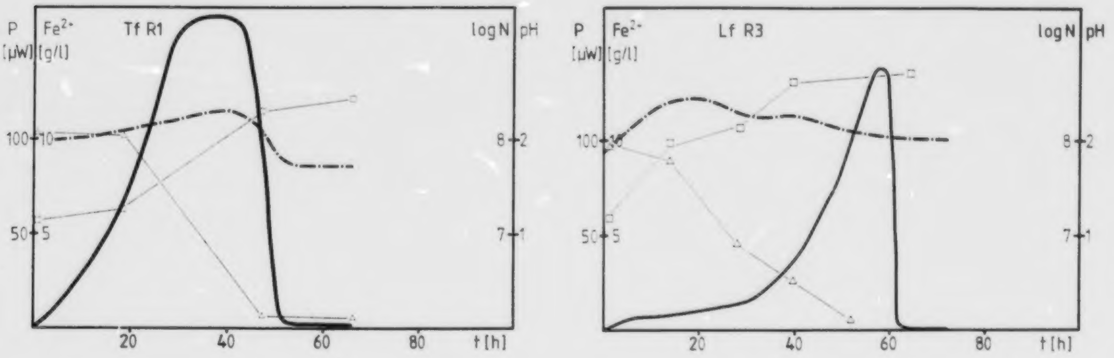

Fig. 5 Thermograms of *T. ferrooxidans* (Tf) R1 and *L. ferrooxidans* (Lf) R3 during growth on 180mMol ferrous iron. Flow-through mode including aeration (2x20ml/h).

P (μW) = ━━━━━━ Fe^{2+} (g/l) = Δ ——— Δ
pH = ━ · ━━ · ━ log N (cells/ml) = □ ——— □

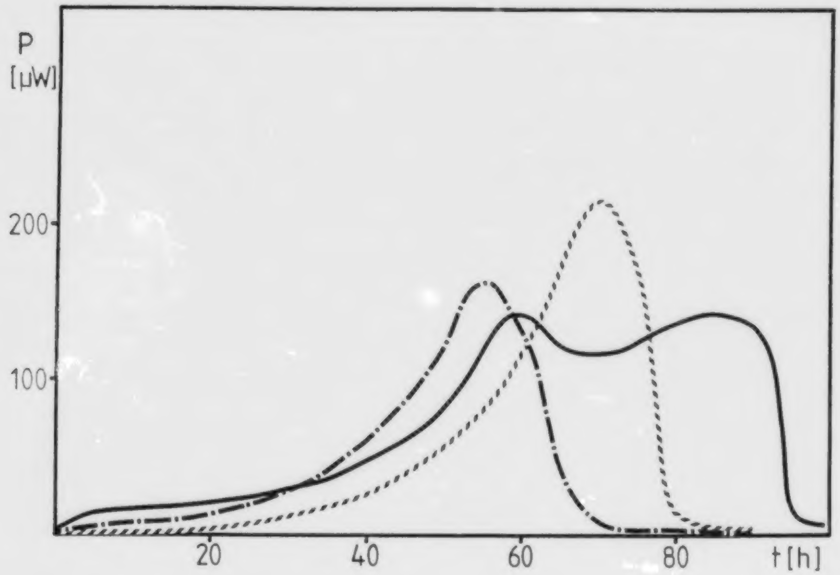

Fig. 6 Thermograms of pure and mixed cultures during ferrous iron oxidation. Flow-through mode including aeration (2x20ml/h).

T. ferrooxidans ATCC 19859 = ━ · ━━ · ━
L. ferrooxidans AS 1 = ╍╍╍╍╍╍
Tf ATCC 19859 + *Lf* AS 1 = ━━━━━━

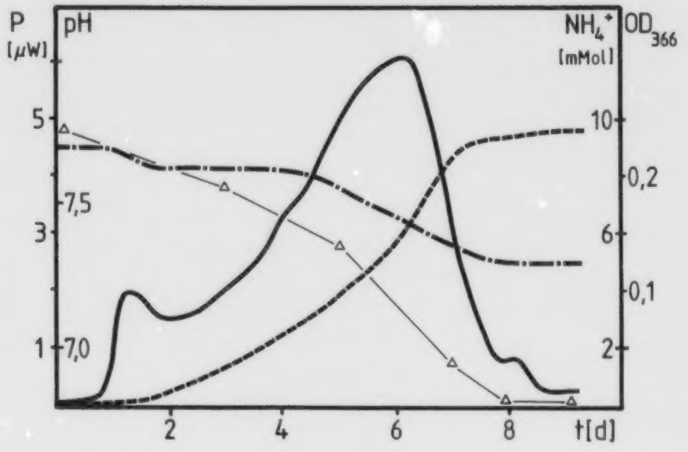

Fig. 7 Thermogram of Nitrosovibrio spec. growing on ammonium chloride (10mMol). Batch culture, flow-through mode including aeration (2x20ml/h), 30°C, HEPES-buffer pH 7.9.

P (µW) = ━━━━━━━━

pH = ▬ ▪ ▬▬▬ ▪ ▬

OD_{366} = - - - - - - - - -

NH_4^+ (mMol) = Δ ———— Δ

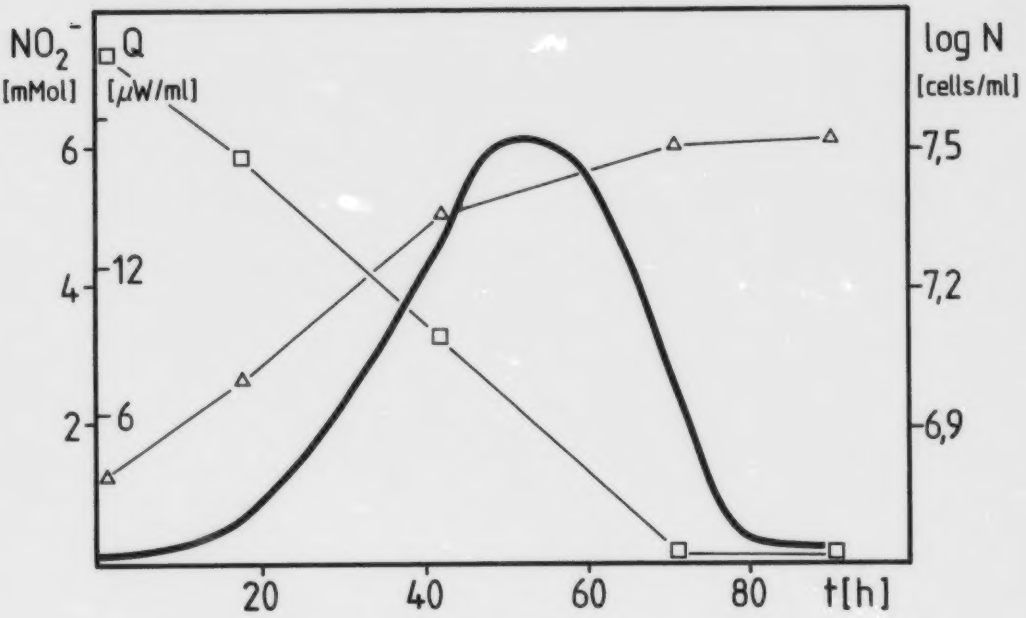

Fig. 8 Thermogram of lithoautotrophic nitrite oxidation (7.5mMol) by *Nitrobacter winogradskyi* ATCC 255. Flow-through mode including aeration, 30°C.

Q (µW/ml) = ━━━━━━━━

log N (cells/ml) = Δ ———— Δ

NO_2^- (mMol) = □ ———— □

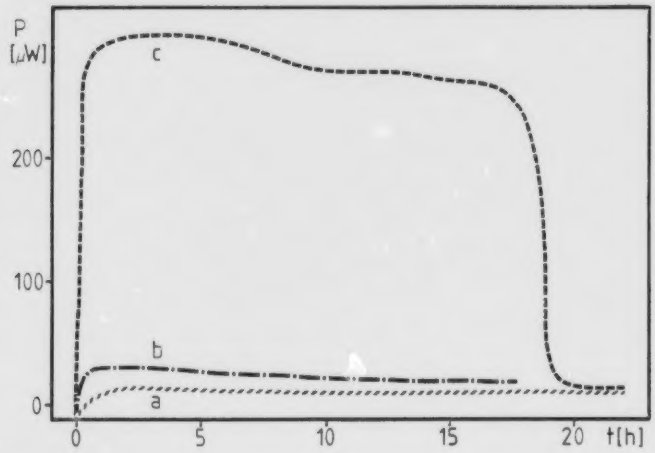

Fig. 9 Thermogram of ferrous iron oxidation (180mMol) by T. ferrooxidans ATCC 19859 (Tf) in the perfusion vessel under aerobic conditions.

a = control with sterile nutrient solution
b = control with Tf in basal salts solution
c = Tf in nutrient solution

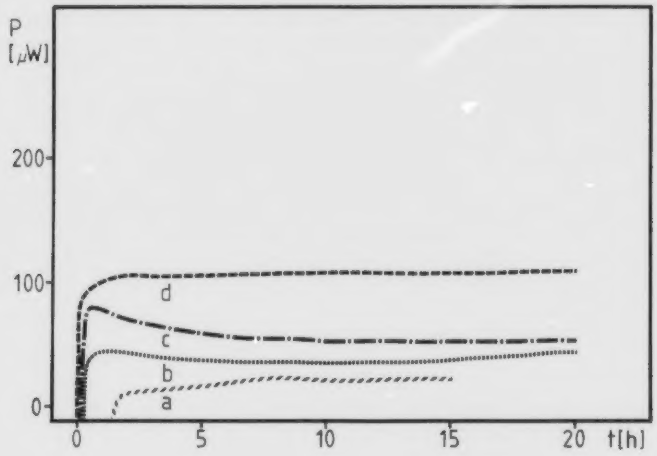

Fig. 10 Thermograms of microbiological pyrite and sulfidic ore oxidation in the perfusion vessel under aerobic conditions. The experiments were made with basal salts solution and addition of 43mg pyrite (a, b, d) or 50mg sulfidic ore (c).

a = control with sterile nutrient solution
b = *T. ferrooxidans* R7
c = *T. ferrooxidans* ATCC 19859
d = *T. ferrooxidans* ATCC 19859

MOLECULAR ASPECTS OF THE STRESS RESPONSE IN *Thiobacillus ferrooxidans* AND OTHER BIOMINING MICROORGANISMS.

*Sergio Alvarez and Carlos A. Jerez**

Departmento de Bioquimica, Facultad de Medicina,
Universidad de Chile, Casilla 70086, Santiago-7, Chile.

ABSTRACT

Bioleaching microorganisms are subjected to very low pH, the presence of toxic metals and temperature variations ranging from freezing to 50°C or higher. Therefore they are good models to study some aspects of the bacterial responses to changes in their environment.

We analyzed the heat shock response in the mesophyllic acidophilic *Leptospirillum Ferrooxidans* (shift from 30°C to 40°C), the moderate thermophile strain LM2 (shifted from 50°C to 60°C) and compared these results with our previous data for *Thiobacillus ferrooxidans* and the thermophilic *Sulfolbus acidocaldarius*. All microorganisms studied showed a heat shock response characterized by an inhibition of general protein synthesis with a concomitant induction of the synthesis of variable number of heat shock proteins. On the other hand, *T. ferrooxidans* did not show a cold shock response (shifted from 30°C to 10°C) as has been described for other bacteria. Interestingly, *T. ferrooxidans* acquired thermotolerance to an otherwise lethal 50°C heat shock when it was previously subjected to a 40°C shift.

The presence of UO_2^{+2} (5 mM), Cu^{+2} (100 mM) or Cd^{+2} (100 mM) did not induce a stress-like response in *T. ferrooxidans*. However ethanol (4%) and a pH shift (3.5 to 1.5) at 30°C elicited a heat shock-like response in this microorganism.

These studies may contribute to an understanding of the molecular basis of response and adaptation of bioleaching bacteria to their habitat.

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*: Corresponding author

ASPECTS MOLÉCULAIRES DE LA RÉPONSE AU STRESS CHEZ *THIOBACILLUS FERROOXIDANS* ET CHEZ D'AUTRES MICRO-ORGANISMES UTILISÉS POUR L'EXTRACTION MINIÈRE

*Sergio Alvarez et Carlos A. Jerez**

Département de Biochimie, Faculté de Médecine
Université de Chili, Santiago, Chili

RÉSUMÉ

Les micro-organismes responsables de la biolixiviation sont sujets à un pH très faible, à la présence de métaux toxiques et à des températures s'échelonnant entre le point de congélation et plus de 50 °C. Par conséquent, ce sont de bons modèles pour l'étude de quelques aspects des réponses bactériennes aux changements dans leur environnement.

Nous avons analysé la réaction au choc thermique d'une souche mésophile et acidophile, *Leptospirillum ferrooxidans,* par un passage de 30°C à 40°C et d'une souche modérément thermophile, LM2, par un passage de 50°C à 60°C. Nous avons comparé ces résultats avec nos données précédentes obtenues *Thiobacillus ferrooxidans* et d'une bactérie thermophile, *Sulfolbus acidocaldarius.* Tous les micro-organismes étudiés ont réagi au choc thermique par une inhibition générale de la synthèse des protéines accompagnée d'une amorce concomitante de synthèse d'un nombre variable de protéines résistantes aux chocs thermiques. Par ailleurs, *T. ferrooxidans* n'a pas réagi à une baisse de température (passage de 30°C à 10°C) comme c'est le cas pour d'autres bactéries. Curieusement, après avoir été soumis à une variation de température de 40°C, *T. ferrooxidans* a développé une tolérance à un choc thermique de 50°C qui autrement aurait été létal.

La présence de UO_2^{+2} ($5\mu M$), de Cu^{+2} ($100\mu M$) ou de Cd^{+2} ($100\mu M$) n'a pas provoqué de réaction au stress chez *T. ferrooxidans* . Toutefois, la présence d'éthanol (4 %) et une variation du pH de 3,5 à 1,5 à 30°C, ont provoqué la même réaction qu'un choc thermique chez ce micro-organisme. Ces études peuvent nous aider à comprendre le fondement moléculaire de la réaction et de l'adaptation des bactéries de lixiviation à leur habitat.

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*Correspondant

INTRODUCTION

The heat shock response is a universal defense mechanism in which a short exposure of the cells to elevated temperatures or other stress agents reduces the genetic expression of the normal cellular proteins, inducing at the same time a transient overproduction of the so-called heat shock (HSPs) or stress proteins, depending on the inducing agent (Neidhart *et al.*, 1984, Lindquist, 1986, Lindquist and Craig, 1988). In *Escherichia coli*, a cold shock which induces the synthesis of several proteins has also been described (Jones *et al.*, 1987).

Several of the acidophilic microorganisms which participate in the bioleaching of minerals are subjected to different kinds of natural environmental stress such as temperature changes, presence of some toxic heavy metals or pH changes which normally take place on bioleaching dumps and that may affect the activity of the bacteria (Brierley, 1978, Tuovinen and Kelly, 1972, Lundgren and Silver, 1980). Ambient temperatures in underground mines are below the optimum range for the mesophyllic *Thiobacillus ferrooxidans*, which has an optimum temperature in the range of 25°C to 35°C (Brierley, 1978, Lundgren and Silver, 1980). Temperatures in underground mines vary from 5°C to 15°C, whereas surface temperature in heap leaching typically vary from below-freezing to up to 50°C or even higher (Kelley and Tuovinen, 1988). It was of interest therefore to study the heat shock response in these important biomining microorganisms. We found previously that *T. ferrooxidans* and *Sulfolbus acidocaldarius* showed a typical heat shock response when transferred from 30°C to 41°C and from 70°C to 85°C respectively (Jerez, 1988, Jerez *et al.*, 1988).

Many kinds of cells acquire a transient thermotolerance when subjected to a heat shock (Neidhart *et al.*, 1984, Lindquist, 1986, Lindquist and Craig, 1988). The heat shock proteins appear to be required for thermotolerance in *Escherichia coli*, and it has been suggested that these cells grown at 45°C have a permanently increased thermal resistance compared to those grown at 30°C (Yamamori and Yura, 1982). This effect might be related to the extraordinary cellular concentrations reached by HSPs at high temperatures (Neidhart *et al.*, 1984, Lindquist, 1986, Lindquist and Craig, 1988).

In the present report we summarize some of the results obtained with *T. ferrooxidans* subjected to different stress agents (temperature, pH, ethanol, heavy metals), extend our results on heat shock to other biomining microorganisms and study in detail the response of *T. ferrooxidans* to different shifts in temperature, from 10°C to 40°C. Finally, we studied the thermotolerance of *T. ferrooxidans* to a 50°C shock with and without a previous heat shock at 40°C.

MATERIALS AND METHODS

Materials.

$Na_2{}^{14}CO_3$ (55 mCi/mmol) and Amplify were purchased from Amersham International. The molecular weight standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Biorad Laboratories and consisted of phosphorylase B (92,500), bovine serum albumin (66,200), ovoalbumin (45,000), carbonic anhydrase (31,000), soy bean trypsin inhibitor (21,500) and lysozyme (14,400).

Bacterial strains and growth conditions.

Thiobacillus ferrooxidans strain DSM 583, the moderate thermophile LM2 and *Sulfolbus acidocaldarius* were a kind gift of Dr. P. Norris. *Leptospirillum ferrooxidans* strains BU-1 and Z-2 were kindly supplied by Dr. A. Harrison. We also employed *T. ferrooxidans* strains ATCC 19859, from the American Type Culture Collection, and strain R2 (Jerez *et al.*, 1986). *T. ferrooxidans* and *L. ferrooxidans* were grown at 30°C in a modified 9K liquid medium (Tuovinen and Kelly, 1973, Jerez *et al.*, 1986) at pH 1.5 or 3.5 where indicated *S. acidocaldarius* was grown at pH 1.7 and 70°C in the presence of $K_2S_4O_6$, as described previously (Norris *et al.*, 1986). The LM2 thermophile was grown as described by Noris and Barr, 1985.

For determination of thermotolerance, *T. ferrooxidans* cells (1.9 x 10^7) were incubated in 9K medium at either 30°C for one hour and then at 50°C for 30 more min. After these heat shock treatments, about 3.8 x 10^4 cells were plated in agarose plates as described by Harrison, 1984, and were all incubated for about 10 days at 30°C to determine the survivors. This was done qualitatively by comparing the oxidation of the plates.

Stress and labelling condition.

Exponentially growing cells of each kind of microorganism were harvested by centrifugation, washed three times in 0.01 N H_2SO_4 and were resuspended at a density of about 5 x 10^9 cells/ml in fresh medium (0.5 ml). About the same number of *S. acidocaldarius* cells were employed, except they were not centrifuged prior to use. The bacterial suspensions were always preincubated for 30 min at their growth temperature. After this time, the control samples were incubated for 60 more min at the same temperature and the experimental samples were shifted to 40-41°C (*T. ferrooxidans* and *L. ferrooxidans*) 50°C (LM2) or 85°C (*Sulfolbus*) for 60 min (heat shock). Alternatively, the *T. ferrooxidans* cells were shifted to 20°C or 10°C (cold shock). After this point, between 4 to 8 uCi of $Na_2{}^{14}CO_3$ (55 mC/mMol) were added to each sample and incubation continued in the presence of the isotope for 30 to 60 min in sealed vessels (Jerez, 1988, Jerez *et al.*, 1988). To test for the effect of ethanol, after the 30 min preincubation ethanol was added (4% final) to the cells which were then incubated at their normal growth temperature for a total of 90 min before the addition of the radioactive carbonate.

To test for the effect of heavy metals, after the 30 min preincubation the appropriate amounts of metals (as sulfates) were added to the cells. For the pH shift, *T. ferrooxidans* cells previously grown at pH 3.5 were centrifuged and resuspended in the same volume of fresh medium at pH 1.5. The cells subjected to these different stress conditions were then incubated at 30°C with radioactive sodium carbonate as before.

Total incorporation of radioactivity into proteins and other components was determined as the hot trichloroacetic acid insoluble material (Jerez *et al.*, 1988).

Polyacrylamide qel electrophoresis of proteins.

The samples, treated as described above, were washed two times by centrifugation with 200 ul of 50 mM Tris-HC1, pH 8, 1 mM EDTA. Finally, the cells were treated with lysozyme and DNAse (Jerez *et al.*, 1986) for 5 min and were boiled in Laemmli sample buffer (Laemmli, 1970). Unless stated otherwise, the same amount of radioactivity was applied to each gel lane. When cells from *T. ferrooxidans* and *L. ferrooxidans* incorporate $^{14}CO_2$, not only the proteins are labelled but also all other cellular components, of which the lipopolysaccharides enter the SDS-PAGE gels (Hitchcock and Brown, 1983, Jerez *et al.*, 1986, Arredondo *et al.*, 1988, Arredondo and Jerez, 1989). To confirm that in our conditions the labelled components separated by SDS-PAGE corresponded to proteins, we subjected the samples to the action of proteinase K before the electrophoretic run (Hitchcock and Brown, 1983, Jerez *et al.*, 1986). Electrophoresis was performed on 10% of 7 to 15% linear sodium dodecyl sulfate-polyacrylamide gradient gels as described previously and after electrophoresis, all the gels were processed for staining and fluorography using Amplify as before (Acuña *et al.*, 1986, Jerez *et al.*, 1988).

RESULTS

The heat shock response in different biomining microorganisms.

We sound that the mesophyllic *T. ferrooxidans* showed a typical heat shock response when shifted from 30°C to 41°C, since this treatment inhibited general protein synthesis and at the same time induced at least ten heat shock proteins (HSPs) (Jerez, 1988, Jerez *et al.*, 1988). We also found that the thermophilic archaebacteria *Sulfolbus acidocaldarius*, which grows at 70°C also showed a heat shock response when shifted to 85°C (Jerez, 1988). To test for the presence of a heat shock response in other bioleaching microorganisms, we used the mesophyllic iron-oxidizer *Leptospirillum ferrooxidans* and the moderate thermophile strain LM2 (Norris and Barr, 1985). Fig. 1 shows the results obtained compared with those for *T. ferrooxidans* and *S. acidocaldarius*. *L. ferrooxidans* showed a response very similar to that of *T. ferrooxidans*. LM2 was apparently very sensitive to a 60°C heat shock, since the total incorporation of $Na_2{}^{14}CO_3$ was greatly reduced and only one possible heat shock protein could be seen under the conditions employed. All analyzed species showed heat shock proteins in the molecular weight range between 50 and 90 KDa.

These results, together with the previous ones suggest that the heat shock response is a general phenomenon in bioleaching microorganisms.

Effect of low temperature shifts on protein synthesis by T. ferrooxidans.

Bacteria are affected by a sudden cold shock (Jones *et al.*, 1987, Ingraham, 1987. Since many bioleaching operations are covered with snow during certain periods of their operation, it was of interest to test for the existence of cold shock in *T. ferrooxidans*. Fig. 2B shows that the total incorporation of $Na_2{}^{14}CO_3$ into hot trichloroacetic insoluble material as already seen, decreases 60% with a heat shock at 40°C. In a similar fashion, when *T. ferrooxidans* cells were shifted from 30°C to 20°C or to 10°C, there was a decrease in the CO_2 fixation of 33% and 75% respectively.

However, when we analyzed the proteins synthesized by the bacteria during the temperature shifts (Fig. 2A), we found that only the 40°C heat shock induced the synthesis of several proteins. The lower temperature shifts during the period tested did not elicit a stress or a cold shock response as the once described for *E. coli* (Jones *et al.*, 1987). The lower temperature limit for the oxidation of iron by *T. ferrooxidans* has recently been described as 4°C (Ahonen and Tuovinen, 1989). Therefore, a temperature lower then 10°C should be tested as a stress for *T. ferrooxidans*.

Induction of thermotolerance in T. ferrooxidans.

To determine whether heat shock at 40°C, in which HSPs are produced, could induce thermotolerance and protect *T. ferrooxidans* cells from the lethal effects of a higher temperature heat shock, the following experiment was carried out. A *T. ferrooxidans* culture was grown at 30°C and after collecting the cells from the lethal effects of a higher temperature heat shock, the following experiment was carried out. A *T. ferrooxidans* culture was grown at 30°C and after collecting the cells, equivalent numbers of microorganisms were 1) kept at 30°C for 120 min, 2) shifted to 40°C for 60 min, then to 30°C for 60 more min, 3) shifted to 50°C for 30 min and then to 30°C for 50 min, and 4) shifted first to 40°C for 60 min, then the same time at 30°C followed by a heat shock of 50°C for 30 min. After these treatments, appropriate numbers of cells were plated and incubated at 30°C as described in Materials and Methods. Fig. 3 clearly shows that a temperature shift of *T. ferrooxidans* cells from 30°C to 40°C does not greatly affect their growth and iron oxidation capacity when qualitatively compared with the control (30°C). On the other hand, as expected, a temperature shift to 50°C is definitively prohibitive to growth. However, when the microorganisms were previously treated at 40°C, they were greatly protected from the lethal effects of the 50°C heat shock.

These data suggest that the synthesis of HSPs (Figs. 1 and 2) by the cells incubated at 40°C induces thermotolerance. In this regard, induction of thermotolerance has been observed in other bacteria and has been attributed to protection afforded by the HSPs (Yamamori and Yura, 1982, Neidhardt *et al.*, 1984, Nelson and Killeen, 1986).

Other stress response inducers.

The induction of some of the heat shock proteins by an external pH change has been described very recently for *E. coli* (Taglicht *et al.*, 1987). When *T. ferrooxidans* cells grown at pH 3.5 were shifted to pH 1.5, there was a response similar to the one observed after heat shock, since several proteins with the same apparent molecular weights were induced in each case (Fig. 4, arrows). Ethanol, at a 4% concentration, is known to exert a response similar to a heat shock in bacteria and several other organisms (Neidhardt *et al.*, 1984). As we have previously shown (Jerez, 1988), this solvent also elicits a similar response in *T. ferrooxidans* grown at 30°C (Fig. 4). This phenomena should be considered, especially when organic solvents are employed for metal extraction in bioleaching operations. The repression of normal protein synthesis by pH change and ethanol was less pronounced compared with the inhibition after heat shock.

Cadmium at a concentration of 600 uM, has been described as inducing many of the HSPs in *E. coli* (Van Bogelen *et al.*, 1987). Since chemolithotrophic bioleaching microorganisms normally grow in the presence of high concentrations of metal ions, it was of interest to analyze the effect of Cd^{+2} and other ions upon *T. ferrooxidans*. Fig. 4 shows that transfer of the bacteria to a medium contain 100 mM Cd^{+2} or Cu^{+2} or 5 mM UO^{+2} did not alter the protein labelling pattern in *T. ferrooxidans*, except for the apparent induction of only one protein band of about 28 kDa in the presence of Cd ion (arrowhead).

The resistance of *T. ferrooxidans* to high metal ion concentrations may reflect some species-specific properties to which general principles concerning metal toxicity may not apply (Tuovinen and Kelly, 1972). In addition, due to the high proton ion concentrations present in the normal *T. ferrooxidans* habitat, the effective metal ion concentrations may need to be much higher.

A summary of the induced proteins found in *T. ferrooxidans* after subjecting the cells to different stress conditions is shown in Fig. 5.

CONCLUSIONS

1. Several biomining microorganisms, from mesophyllic to thermophilic, show a typical heat shock response which is elicited at a temperature which varies depending on the normal growth temperature of the corresponding microorganism.
2. Cold temperature shifts (30°C to 20°C or 10°C) for 90-120 min do not elicit a cold shock response in *T. ferrooxidans*.
3. Thermotolerance to 50°C is induced in *T. ferrooxidans* by a previous non-lethal heat shock (40-41°C).
4. Toxic metals known to induce a heat shock-like response in other organisms do not affect *T. ferrooxidans*.
5. A pH shift from 3.5 to 1.5 induces a heat shock-like response in *T. ferrooxidans*.
6. An organic solvent such as ethanol, also provokes a heat shock-like response in *T. ferrooxidans*.

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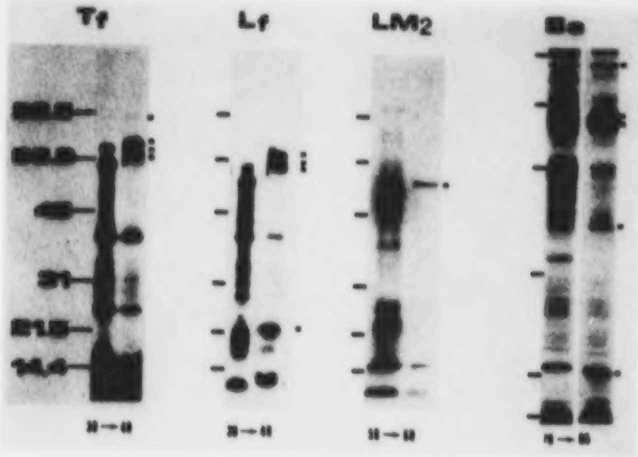


Fig. 1 Heat shock response in different biomining microorganisms. Cells of *T. ferrooxidans* (Tf), *L. ferrooxidans* (Lf), strain LM2 (LM2) and *S. acidocaldarius* (Sa) were shifted from their respective growth temperatures to the indicated shock temperatures for 90 min in the presence of $Na_2{}^{14}CO_3$ and the radioactive proteins were analyzed by SDS-PAGE and autoradiography. Numbers to the left indicate molecular weight standards in KDa. The induced heat shock proteins are indicated by solid squares.

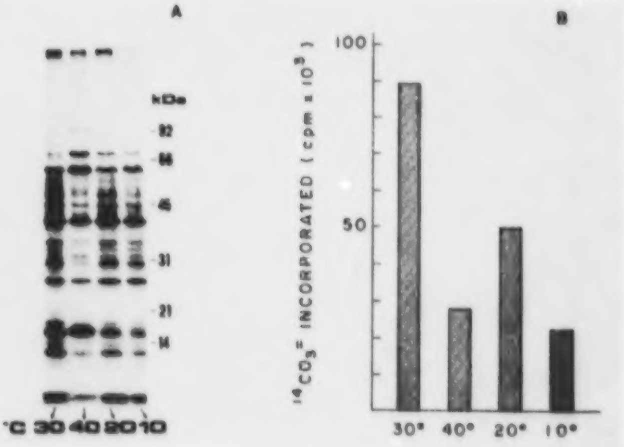


Fig. 2 Effect of different temperature shifts on $Na_2{}^{14}CO_3$ incorporation into proteins by *T. ferrooxidans*. Cells were incubated at 30°C or were transferred from this temperature to the indicated temperatures in the presence of $Na_2{}^{14}CO_3$. The radioactively labelled proteins were analyzed by SDS-PAGE- and autoradiography (A) and the total incorporation of radioactivity into trichloroacetic insoluble material was also measured (B).

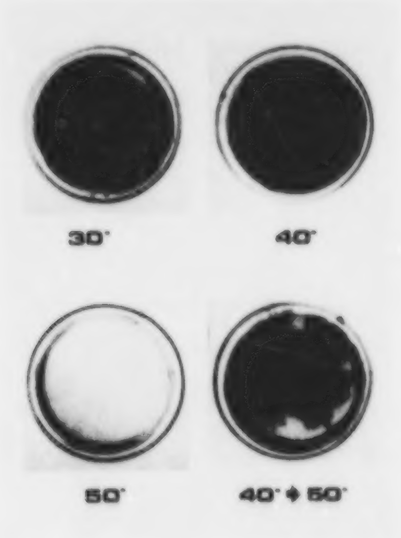


Fig. 3 Thermotolerance of *T. ferrooxidans* induced by heat shock. *T. ferrooxidans* cells were treated at the indicated temperatures for 60 min (30°C and 40°C) or 30 min (50°C). Alternatively, the bacteria were treated first at 40°C for 60 min, returned to 30°C for the same time, and then heat-shocked at 50°C for 30 more min (bottom right plate). After these incubations, the same amount of each kind of cells was plated and were all incubated at 30°C for 10 days.

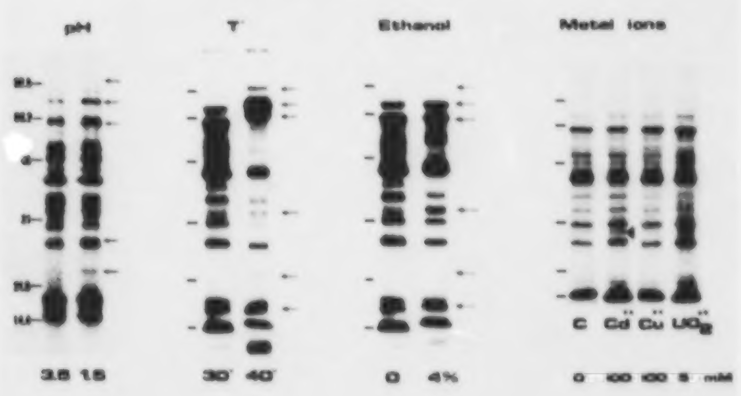
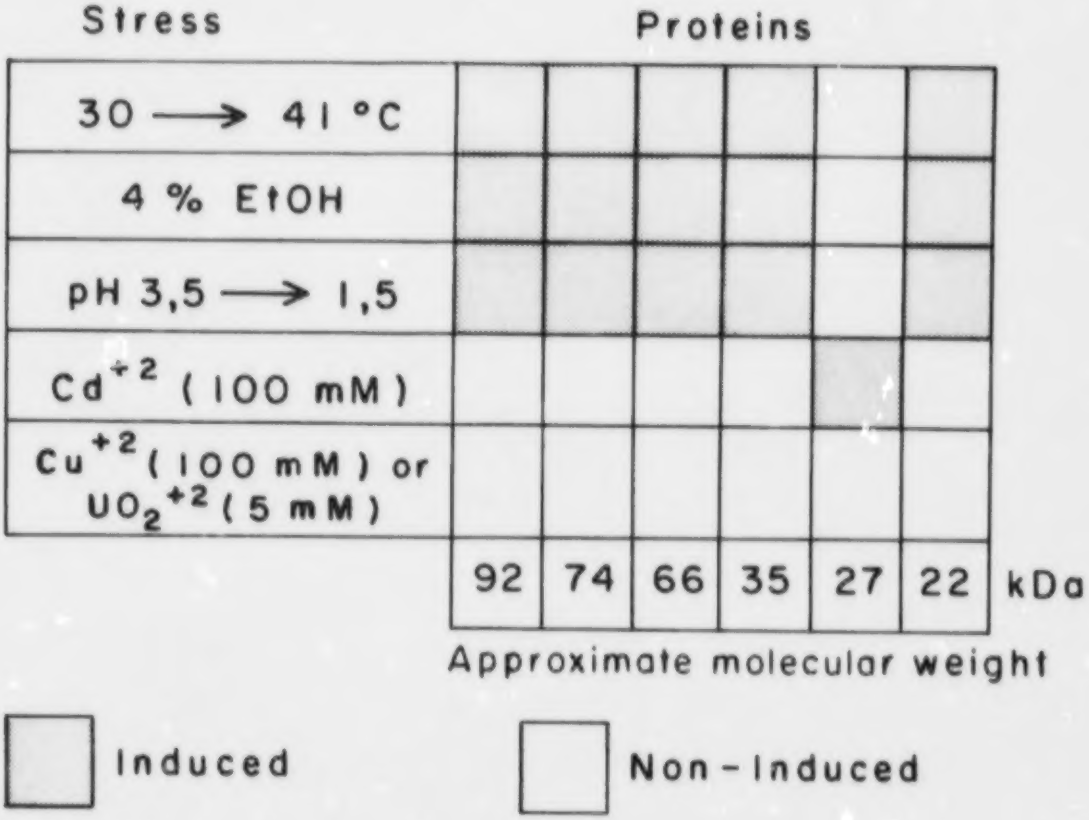


Fig. 4 Effect of different stress conditions on protein synthesis by *T. ferrooxidans*. Cells were labelled with $Na_2{}^{14}CO_3$ under the indicated conditions of pH, temperature and presence or absence of ethanol or metal ions. The proteins synthesized during these treatments were analyzed by SDS-PAGE and autoradiography as described in Materials and Methods. Numbers to the left indicate molecular weights of markers in KDs and arrows or arrowhead indicate the main proteins induced in each case.



Stress	Proteins					
30 ⟶ 41 °C	☐	☐	☐	☐	☐	▨
4 % EtOH	▨	▨	▨	☐	☐	▨
pH 3,5 ⟶ 1,5	▨	▨	▨	▨	☐	▨
Cd^{+2} (100 mM)	☐	☐	☐	☐	▨	☐
Cu^{+2} (100 mM) or UO_2^{+2} (5 mM)	☐	☐	☐	☐	☐	☐
	92	74	66	35	27	22 kDa

Approximate molecular weight

▨ Induced ☐ Non-Induced

Fig. 5 Summary of proteins induced in *Thiobacillus ferrooxidans* by different stress conditions.

THE REGULATION OF SULFUR UTILIZATION BY FERROUS ION IN *THIOBACILLUS FERROOXIDANS*

by

T. Sugio, T. Hirose, A. Oto, K. Inagaki, T. Tano

Division of Biological Function and Genetic Resources Science,
Faculty of Agriculture, Okayama University, Okayama 700, Japan.

ABSTRACT

The utilization of elemental sulfur($S°$) as an energy source by *T. ferrooxidans* AA19-3 was strongly inhibited by above 0.108 M Fe^{2+}. The Fe^{2+} (0.108 M) added to $S°$-salts medium was rapidly oxidized enzymatically by iron oxidase of the cells at the early stage of cultivation, but approximately 0.6 mM Fe^{2+} remained in the medium without being oxidized for 30-day cultivation. In a similar way, the utilization of sulfur in a copper concentrate by this strain and the amount of Cu^{2+} solubilized from the ore were strongly inhibited by above 0.108 M Fe^{2+}. The mechanisms by which Fe^{2+} inhibits *T. ferrooxidans* AP19-3 use of sulfur as an energy source were studied, and two causes for this inhibition were clarified. We have proposed a new sulfur oxidation route in this strain, in which $S°$ is oxidized by the cooperation of three enzymes, namely: sulfur:ferric ion oxidoreductase(SFORase), sulfite:ferric ion oxidoreductase, and iron oxidase, and Fe^{3+} and Fe^{2+} act as mediators of electrons among these enzymes. The SFORase and sulfite:ferric ion oxidoreductase catalyze $S°$ and sulfite oxidations with Fe^{3+} as an electron acceptor, respectively. It was found that the activities of both SFORase and sulfite:ferric ion oxidoreductase were completely inhibited by 20 mM and 1 mM Fe^{2+}, respectively, indicating that Fe^{2+} inhibits two important enzymes of this strain involved in sulfur oxidation to stop the cells utilizing sulfur as an energy source. A sulfite completely inhibited cell growth on $S°$-salts medium at 0.5 mM. In contrast, cell growth on Fe^{2+}-salts medium was not affected by 5 mM sulfite. The specific activity of SFORase of the cells incubated with both $S°$ and 0.108 M Fe^{2+} for 4 days decreased to 58% of that without Fe^{2+}, suggesting that the sulfite, inevitably accumulated in the cells when a sulfite:ferric ion oxidoreductase is inhibited by Fe^{2+}, causes damage to the cells, in particular, to the SFORase.

RÉGULATION DE L'UTILISATION DU SOUFRE PAR DES IONS FERREUX CHEZ *THIOBACILLUS FERROOXIDANS*

T. Sugio, T.Hirose, A. Ota, K.Inagaki et T. Tano

Division des Ressources Génétiques et des Fonctions Biologiques
Faculté d'Agriculture, Université Okayama, Okayama, Japon

RÉSUMÉ

L'utilisation du soufre élémentaire (S^0) comme source d'énergie par *T. ferrooxidans* AP19-3 a été fortement inhibée par plus de 0,108 M de Fe^{2+}. Le $Fe2^+$ (0,108 M) ajouté à un milieu constitué de sels de S^0 a été rapidement oxydé enzymatiquement par l'oxydase de fer de cellules au stade initial de développement, mais environ 0,6μM de Fe^{2+} n'a pas été oxydé dans une culture de 30 jours. De la même manière, l'utilisation par cette souche du soufre dans un concentré de cuivre et la solubilisation du Cu^{2+} dans le minerai ont été fortement limitées par plus de 0,108 M de Fe^{2+}. Le mécanisme par lequel le Fe^{2+} empêche *T. ferrooxidans* AP19-3 d'utiliser le soufre comme source d'énergie a été étudié et les deux causes de cette inhibition ont été clarifiées. Nous avons proposé une nouvelle méthode d'oxydation du soufre (S^0) par cette souche, qui fait appel à trois enzymes, notamment l'oxydoréductase ion ferrique: soufre, (SFORase) l'oxydoréductase d'ion ferrique: sulfite et l'oxydase du fer, le Fe^{3+} et le Fe^{2+} agissent comme médiateurs d'élection entre ces enzymes. La SFORase et oxydoréductase ion ferrique: sulfite catalysent le S^0 et l'oxydation du sulfite, en présente du Fe^{3+} agissant comme accepteur d'électron. Il a été trouvé que l'activité de la SFORase et de l'oxydoréductase ion ferrique: sulfite a été complètement inhibé par 20 mM et 1mM de Fe^{2+}. Ceci indique que le Fe^{2+} inhibe deux enzymes importantes de certe souche, impliquées dans l'oxydation du soufre, empêchant alors les cellules d'utiliser le soufre comme source d'énergie. Le sulfite inhibe complètement la croissance de la cellule en milieu constitué de sels de S^0 à une concentration de 0.5 mM. Au contraire, cette croissance cellulaire sur un milieu composé de sels de $Fe^{2}+$, n'était pas affecté par 4mM de sulfite. L'activité spécifique de la SFORase des cellules incubeés pendant 4 jours avec le S^0 et 0.108M de Fe^{2+} a baisseé à 58% de celle en absence de Fe^{2+}, ce qui suggère que le sulfite s'accumble inévitablement dans les cellules lors que l'oxydoréductase ion ferrique: sulfite est inhibeé par le Fe^{2+}, causant alors des dommages aux cellules, particulièrement à l'enzyme SFORase.

INTRODUCTION

Iron oxidizing bacterium *Thiobacillus ferrooxidans* has been considered one of the most valuable microorganisms for the bacterial leaching of sulfide ores. These abilities of *T. ferrooxidans* are due to their possession of unique enzymes systems that can oxidize both Fe^{2+} and reduced sulfur compounds. By using these abilities, *T. ferrooxidans* has been thought to attack sulfide ores to carry out rapid solubilization of metals. In order to clarify the mechanism of bacterial leaching more precisely, it is important to study whether *T. ferrooxidans* simultaneously attacks both Fe^{2+} and sulfur of sulfide ore regardless of environmental conditions. We found that the utilization of elemental sulfur (S°) as an energy source by *T. ferrooxidans* AP19-3 was strongly inhibited by above 0.108 M Fe^{2+}. The results suggest the possibility that in an environment in which above 0.108 M Fe^{2+} is present, the bacterium cannot use Fe^{2+} and sulfur simultaneously. This prompted us to investigate the mechanism by which Fe^{2+} inhibits the bacterium from using sulfur as an energy source.

Suzuki *et al.* proposed the mechanism of a solid sulfur oxidation in *Thiobacillus thiooxidans* and *thiobacillus thioparus*, in which sulfur dioxygenase plays a crucial role (14, 15). The mechanism of sulfur oxidation by *T. ferrooxidans* has been considered similar to that of other thiobacilli (1,3,4,16). We recently proposed a new sulfur oxidation route in *T. ferrooxidans* AP19-3, in which S° is oxidized by the cooperation of three enzymes, namely, sulfur:ferric ion oxidoreductase (SFORase), sulfite:ferric ion oxidoreductase, and iron oxidase (8,9,11-13). A SFORase, which catalyzes the oxidation of S° with Fe^{3+} as an electron acceptor to give sulfite and Fe^{2+}, was purified to an electrophoretically homogeneous state from iron-grown *T. ferrooxidans* AP19-3 (9). A sulfite:ferric ion oxidoreductase catalyzes the oxidation of sulfite with Fe^{3+} as an electron acceptor to give Fe^{2+} and sulfate (12). We also showed that the sulfite produced as an intermediate during sulfur oxidation by *T. ferrooxidans* AP19-3, if it is not decomposed rapidly enzymatically by iron oxidase or chemically by Fe^{3+}, markedly damages both SFORase and iron oxidase (10).

In this study, we show that the utilization of sulfur as an energy source by *T. ferrooxidans* AP19-3 is strongly inhibited by above 0.108 M Fe^{2+}; Fe^{2} inhibits both sulfur:ferric ion oxidoreductase and sulfite:ferric ion oxidoreductase to stop the cells use of sulfur as an energy source; and the sulfite, inevitably accumulated in the cells when a sulfite:ferric ion oxidoreductase is inhibited by Fe^{2+}, causes damage to the cells.

MATERIALS AND METHODS

Microorganism and Media T. ferrooxidans AP19-3 (6) was used throughout this study. The composition of the basal salts solution used throughout this study was the same as that used by Silverman *et al.*(5). Iron- salts medium was prepared by autoclaving 200 mL of basal salts solution supplemented with ferrous sulfate. The composition of sulfur-salts medium was as follows: 200 mL of basal salts solution; S°, 2 g; and Fe^{3+}, 0.5 mM.

Activities of Sulfur:Ferric Ion Oxidoreductase and Sulfite: Ferric Ion Oxidoreductase. The activity of sulfur:ferric ion oxidoreductase (SFORase) was determined by measuring sulfite in the reaction mixture under aerobic conditions (9). The reaction mixture composed of 4 mL of 0.1 M sodium phosphate buffer (pH 6.5), 0.2 mg of bovine serum albumin, 100 mg of S°, 200 μmol GSH (adjusted to pH 6.5 with dilute NaOH); and a purified SFORase at the stage of Mono Q column chromatography (9). Total volume was 5.0 mL. The sulfite produced was determined colorimetrically by the pararosaniline method (17). The activity of SFORase was also determined with washed intact cells by measuring Fe^{2+} produced in the reaction mixture (7). The presence of Fe^{2+} was determined colorimetrically by a modification of the *o*-phenanthroline method (2).

The activity of sulfite:ferric ion oxidoreductase was determined by measuring the decrease of sulfite in the reaction mixture (12).

RESULTS

Effect of Ferrous Ion on the Utilization of Sulfur as an Energy Source by T. Ferrooxidans AP19-3. Iron grown *T. ferrooxidans* AP19-3 was inoculated into 200 mL of S°(1%)-salts medium supplemented with or without Fe^{2+}, or Fe^{2+}(0.108 M)-salts medium without S°, and cultured by shaking at 30°C. The bacterium grew to a maximum cell growth of 8 x 10^8 cells per mL in S°-salts and iron-salts media, respectively (Fig. 1). The utilization of S° as an energy source by this strain was strongly inhibited by above 0.108 M Fe^{2+} because the amount of cell growth on S°-salts medium supplemented with 0.108 M Fe^{2+} was similar to that observed in Fe^{2+} (0.108 M)-salts medium lacking S°. In contrast, the utilization of Fe^{2+} as an energy source by this strain was not inhibited by 1% S°. After 10-day cultivation in S°-salts medium without Fe^{2+}, in which the bacterium grew 4 x 10^8 cells per mL medium Fe^{2+} was added to the medium at 0.108 M, resulting in an immediate stop of cell growth (data not shown).

Utilization of the sulfur in copper concentrate was found to be similar by this strain. The composition of copper concentrate utilized is as follows: Cu, 20.48%; Fe, 38.22%; Pb, 3.84%; and Zn, 4.22%. Both sulfur utilization and the amount of Cu^{2+} solubilized from the ore were strongly inhibited by above 0.108 M Fe^{2+} (data not shown).

Effect of Fe^{2+} on the Activities of Sulfur:Ferric Ion Oxidoreductase and Sulfite:Ferric Ion Oxidoreductase. The activity of a sulfur:ferric ion oxidoreductase (SFORase) measured with intact cells and a purified SFORase were completely inhibited by 2 mM and 20 mM Fe^{2+}, respectively (Fig. 2). The activities of sulfite:ferric ion oxidoreductase measured with a plasma membrane fraction and the fraction solubilized from plasma membrane fraction and the fraction solubilized from plasma membrane with 1% Nonidet P-40 were completely inhibited by 5 mM and 1 mM Fe^{2+}, respectively. Metal ions, such as cupric, chromium, zinc, cadmium, nickel, barium, and lead did not inhibit a sulfite:ferric ion oxidoreductase at 10 mM (Fig. 2B), suggesting that the activity of sulfite:ferric ion oxidoreductase is specifically regulated by Fe^{2+}.

Ferrous ion added to S°-salts medium at 0.108 M and 0.108 M was rapidly oxidized enzymatically by iron oxidase at the early stage of cultivation. However, 0.6 mM and 1.0 mM Fe^{2+} remained in these media without being oxidized after 30-day cultivation, respectively (Fig. 1). Since a low level of Fe^{2+} strongly inhibits two enzymes involved in S° oxidation of this strain, the Fe^{2+} remaining in the medium may be sufficient to continuously inhibit these enzymes to stop cell use of S° as an energy source.

Decrease of a Specific Activity of Sulfur:Ferric Ion Oxidoreductase During Incubation of T. Ferrooxidans AP19-3 in Fe^{2+} Supplemented Sulfur-Salts Medium. The cells (20 mg protein) grown on iron-salts medium were inoculated into S°-salts medium with or without Fe^{2+} and incubated at 30°C on a reciprocal shaker to check whether SFORase is damaged during incubation with both Fe^{2+} and S°. After a 4-day incubation, cells were collected, washed with 0.1 M ß-alanine buffer (pH 3.0), and SFORase activity was measured with intact cells. The specific SFORase activity of the cells incubated with S° and 0.108 M Fe^{2+}, decreased to 58% of that without Fe^{2+} and the decrease in specific activity was proportional to the concentration of Fe^{2+} added to incubation mixture (Fig. 3).

Cell growth on iron-salts medium was not affected by 5 mM sulfite. In contrast, a sulfite completely inhibited cell growth on S°-salts medium at 0.5 mM, indicating that sulfite is more harmful to the cells when the cells grow on S°-salts medium than on iron-salts medium (Fig. 4). Specific SFORase activity of the cells treated with 5 mM sulfite for 1 h markedly decreased (data not shown). These results suggest that the sulfite, inevitably accumulated in the cells when a sulfite:ferric ion oxidoreductase is inhibited by Fe^{2+}, causes damage to SFORase.

CONCLUSIONS

A high concentration of Fe^{2+} (above 0.108 M) added to sulfur (1%)-salts or copper concentrate (1%)-salts media strongly inhibited *T. ferrooxidans* AP19-3 use of sulfur as an energy source. It was found that two important enzymes of this strain involved in sulfur oxidation, sulfur:ferric ion oxidoreductase and sulfite:ferric ion oxidoreductase, are strongly inhibited by Fe^{2+} to stop the bacterium from using sulfur as an energy source. The inhibition of these enzymes by Fe^{2+} is quite reasonable because Fe^{2+} is a common reaction product of these enzyme reactions.

The results obtained in this text suggest that if a high concentration of Fe2+ is present, *T. ferrooxidans* preferentially utilizes Fe2+ as an energy source, but not sulfur. Thus, in the presence of a high concentration of Fe2+, metal ions may be solubilized from sulfide ores mainly by an indirect leaching mechanism. It was also suggested that sulfite or an intermediate during sulfur oxidation of *T. ferrooxidans* AP19- 3 causes damage to the cells, in particular, to SFORase.

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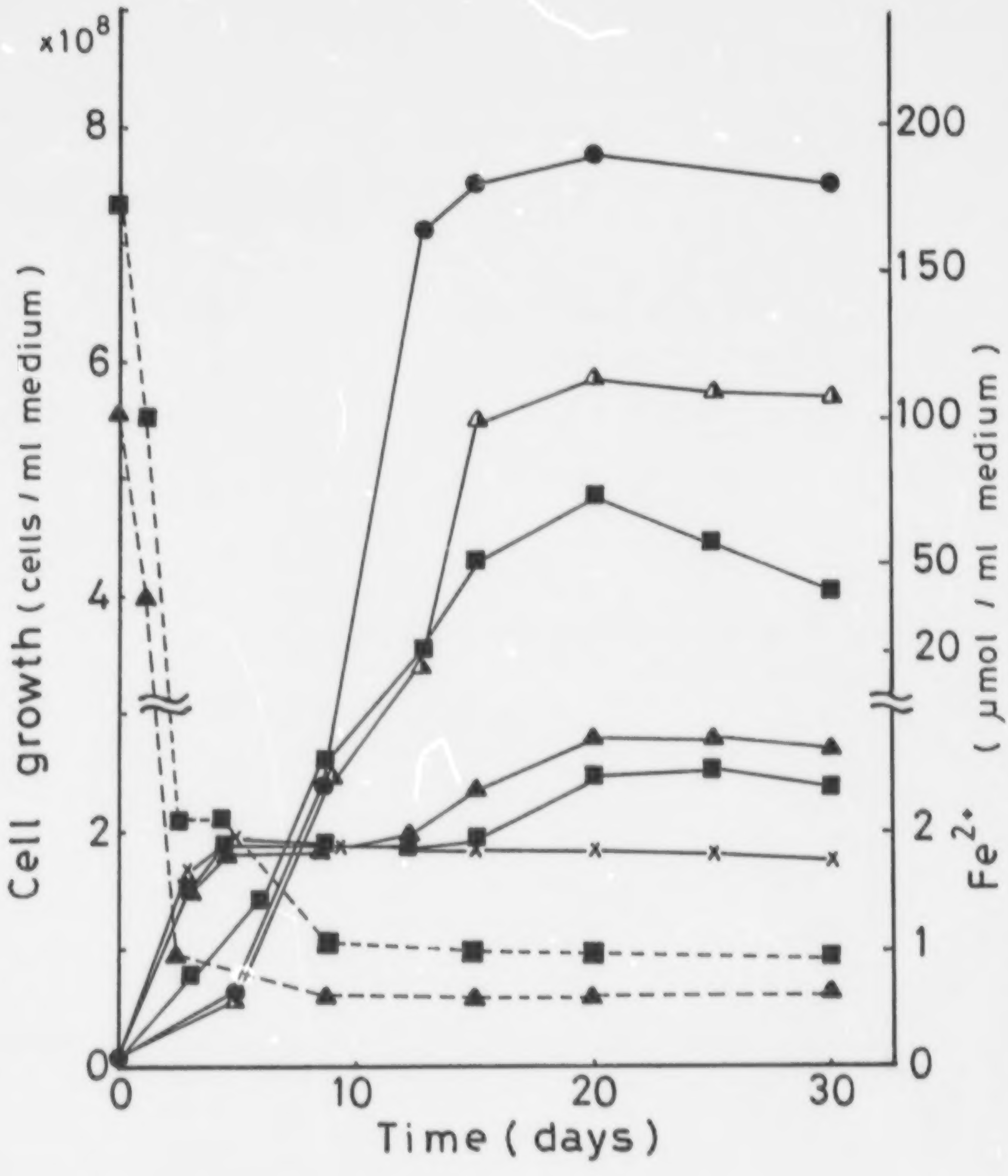

Fig. 1 Effect of ferrous ion on the growth of *T. ferrooxidans* AP19-3 on sulfur (1%)-salts medium. Solid and dotted lines show cell growth and the decrease of Fe^{2+} in the medium, respectively. Symbols: ●, sulfur-salts medium; sulfur-salts medium supplemented with 0.018 M (●), 0.036 M(■), 0.108 M(●) and 0.180 M (■) of Fe^{2+}; Fe^{2+} (0.108 M)-salts medium without sulfur(X)

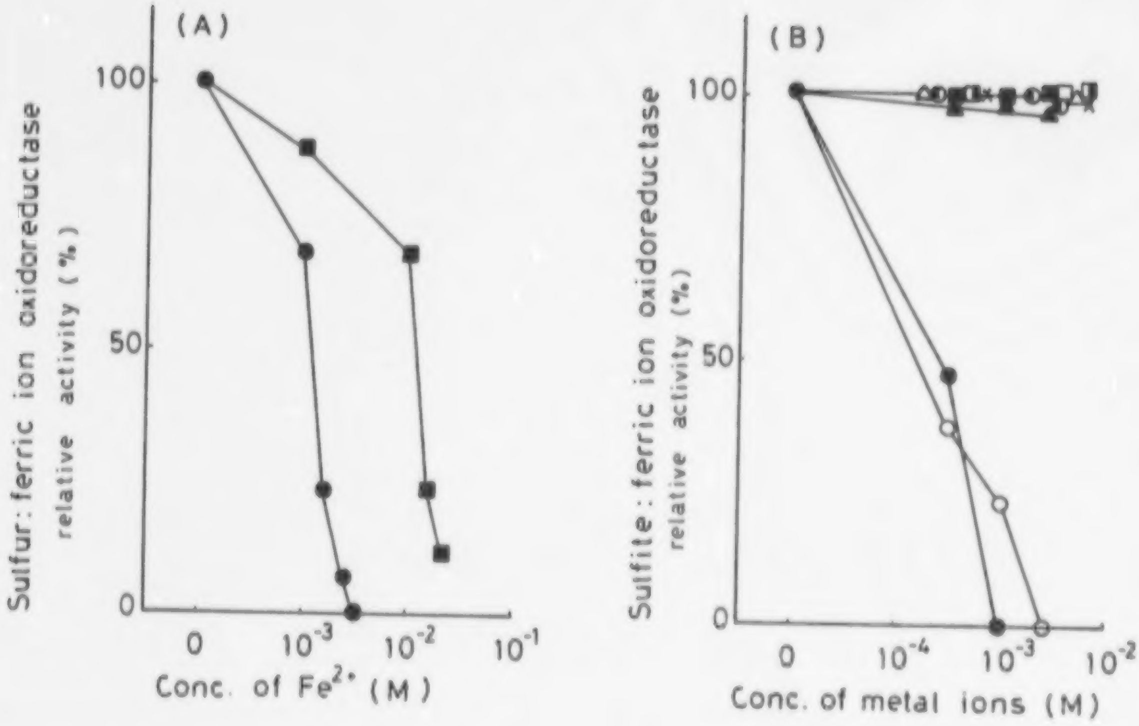

Fig. 2 Effect of ferrous ion on the activities of sulfur:ferric ion oxidoreductase and sulfite:ferric ion oxidoreductase. (A) Effect of Fe^{2+} on the activity of sulfur:ferric ion oxidoreductase (SFORase). Symbols: the activity was measured with washed intact cells(●) or a purified SFORase at the stage of Mono Q column chromatography (■) (9). (B) Effect of Fe^{2+} on the activity of sulfite:Ferric ion oxidoreductase. Symbols: the activity was measured with plasma membrane fraction (○) or the fraction solubilized from plasma membrane with 1% Nonidet P-40(●). Effect of Pb^{2+}(■), Cu^{2+}(□), Cr^{3+}(□), Zn^{2+}(X), Cd^{2+}(●), Ni^{2+}(●), and Ba^{2+}(●) on the activity were also determined with plasma membrane fraction.

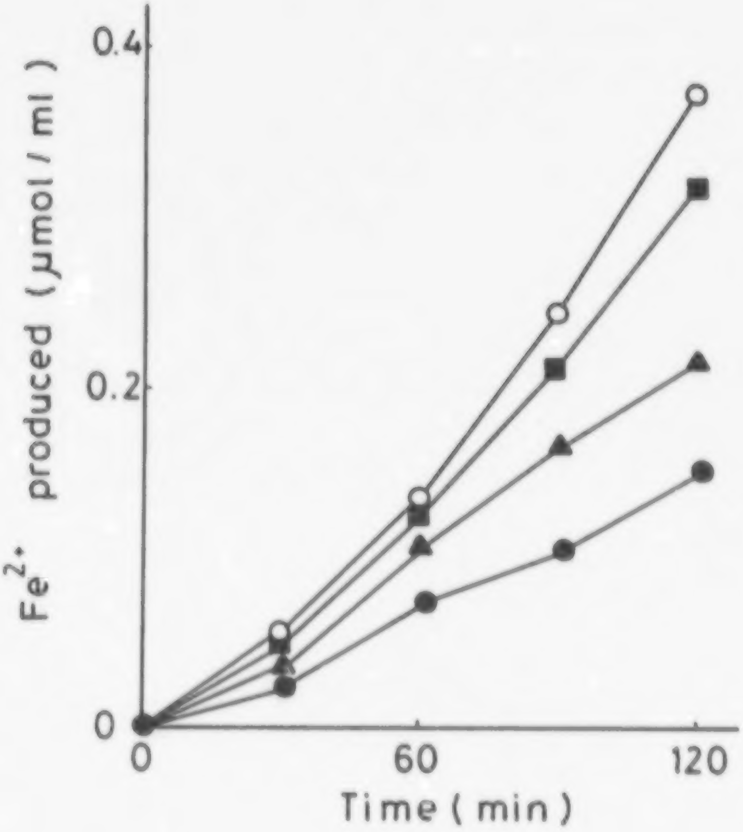

Fig. 3 A specific activity of sulfur:ferric ion oxidoreductase of the cells incubated with sulfur (1%)-Fe^{2+}-salts medium for 4 days. The activity of sulfur:ferric ion oxidoreductase was determined with washed intact cells. Symbols: ○, SFORase activity of the cells incubated with S°-salts medium without Fe^{2+}; SFORase activity of the cells incubated with S°-salts medium supplemented with 0.036 M(■), 0.108 M(■), or 0.180 M(●) of Fe^{2+}.

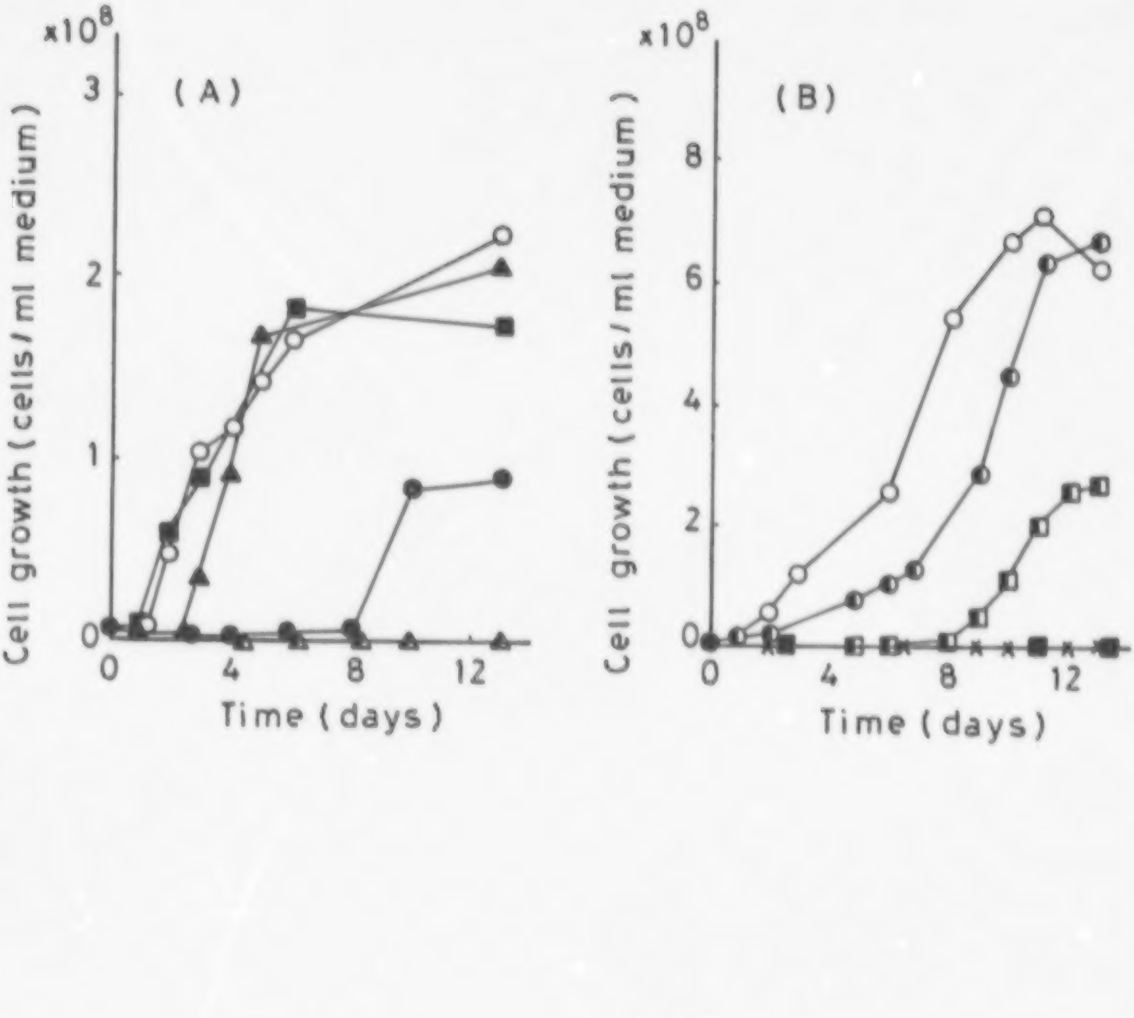

Fig. 4 Effect of sulfite on the growth of *T. ferrooxidans* AP19-3 on iron-salts or sulfur-salts medium. (A) Cell growth on Fe^{2+} (0.108 M)-salts medium supplemented with 1 mM(■), 5 mM(■), 10 mM(●) and 50 mM(●) of sulfite, or without sulfite(○). (B) Cell growth on sulfur (1%)- salts medium supplemented with 1 μM(○), 0.1 mM(■), 0.5 mM(X) and 1 mM (■), or without sulfite(○).

THIOBACILLUS FERROOXIDANS RESISTANCE TO MOLYBDENUM

by

G.I. KARAVAIKO, T.A. PIVOVAROVA, AND R.S. DZHANSUGUROVA
Institute of Microbiology, USSR Academy of Sciences, Moscow

ABSTRACT

Molybdenum is an element highly toxic to *T. ferrooxidans* and inhibits the latter via the non-competitive pathway. An apparent K_m value at pH 1.5 and 2.5 is 1.37 ± 0.4 and 1.25 ± 0.5 mM Fe^{2+} respectively while the inhibition constant K_i is 0.076 mM. However, Mo^{6+} is not resistant in 9K medium containing *T. ferrooxidans* metabolites and is precipitated or chelated depending on its concentration, pH of the medium and Fe^{3+} content, whereby its toxicity decreases. Thus, addition of 20-30% of inoculum to a medium containing 150-500 $mg.l^{-1}$ Mo^{6+} virtually completely eliminates its toxic effect. It is shown that in the presence of molybdenum *T. ferrooxidans* most actively synthesizes such exometabolites as amino acids, nucleotides and organic acids. The highest activity is observed in the production of amino acids which is dominated by the synthesis of dicarboxylic amino acids and amino acids containing additional hydroxyl groups and amino groups.

After the removal of amino acids from the culture liquid the latter completely loses its protective properties. This suggests that amino acids play the principal role in Mo^{6+} chelation.

RÉSISTANCE DE *THIBOBACILLUS FERROOXIDANS* AU MOLYBDÈNE

G.I. Karavaiko, T.A. Pivovarova et R.S. Djansugurova

Institut de Microbiologie, Académie des Sciences de l'U.R.S.S., Moscou

RÉSUMÉ

Le molybdène est un élément hautement toxique pour *T. ferrooxidans* et il provoque une inhibition non-compétitive de ce dernier. Pour des pH de 1,5 et de 2,5 on obtient respectivement des valeurs apparentes de K_m de 1,37 ± 0,4 et de 1,25 ± 0,5 μM Fe^{2+} alors que la constante d'inhibition K_i est de 0,076 μM. Toutefois, le Mo^{6+} ne résiste pas dans un milieu 9K renfermant des métabolites produits par *T. ferrooxidans* et il est précipité ou chélaté selon sa concentration, selon le pH du milieu et selon la teneur en Fe^{3+}, tandis que sa toxicité diminue. Ainsi, l'ajout de 20 à 30 pour cent d'un inoculum à un milieu renfermant de 150 à 500 mg.L-1 de Mo^{6}, élimine presque complètement l'effet toxique de ce dernier. Il est démontré qu'en présence de molybdène, *T. ferrooxidans* synthétise très rapidement des exométabolites comme des acides aminés, des nucléotides et des acides organiques. C'est la production d'acides aminés qui est la plus importante et elle est caractérisée principalement par la synthèse d'acides aminés dicarboxyliques et d'acides aminés renfermant des groupes hydroxyles et amines supplémentaires.

Après extraction des acides aminés du liquide de culture, celui-ci perd complètement ses propriétés protectrices, ce qui suggère que les acides aminés jouent un rôle clé dans la chélation du Mo^{6+}.

INTRODUCTION

Investigations of the effect of molybdenum on *T. ferrooxidans* were initiated in the late 1950's in the context of its leaching from molybdenum ores. It was shown that molybdenum was highly toxic and that bacteria developing in Fe^{2+} containing medium died at Mo concentrations as low as 5-40 mg.l^{-1} (Zimmerley *et al.*, 1958; Bhappu *et al.*, 1965; Kamalov, 1970; Tuovinen *et al.*, 1971; Duncan *et al.*, 1976; Roy A. Mishra, (1981).

Multiple transfers into Fe^{2+} – containing medium with gradually increasing concentrations of molybdenum yielded bacterial strains resistant to 70-200 mg.l^{-1} Mo^{6+} (Kamalov, 1970). However, the rate of Fe^{2+} oxidation was low. In the 1970's Brierley *et al.* (Brierley, 1972; Brierley and Murr, 1973) showed high molybdenum resistance of *Sulfolbus brierleyi* (presently *Acidianus brierleyi*, Segerer *et al.*, 1986). This bacterium was resistant to 2 g.l^{-1} Mo^{6+} when developing in media containing elemental sulfur. However, neither the mechanism of reistance of this organism to Mo^{6+} nor the behaviour of molybdenum in such environment was studied.

We have shown earlier that *T. ferrooxidans* without preadaptation is capable of actively growing in Fe^{2+} – containing media in the presence of 250-500 mg.l^{-1} Mo^{6+} supplemented with 20-30% of inoculum (Karavaiko *et al.*, 1989).

The present investigation aimed at studying the factors behind *T. ferrooxidans* resistance to molybdenum ions.

METHODS

T. ferrooxidans strain VKM B-458 was used in the experiments. The experiments were performed in 300 mL Erlenmeyer flasks in 9K medium containing Fe^{2+} at 28°C under shaking (180 rpm). Molybdenum as ammonium molybdate was added after the inoculation of the medium. The growth of bacteria was measured by protein using the Lowry method while the O_2 uptake rate was determined in the Warburg device of the OH-107 polarograph (Radelkis) using the Clark oxygen cell. K_m and K_i were calculated using the method of Lineweaver-Burk and Hunter-Downs (Dickson and Webb, 1982).

Fe^{2+} and Fe^{3+} were determined complexometrically (Reznikov *et al.* 1970) while molybdenum determinations used the rodanide method with thiourea (Zaichikova, 1949). Since rodanide is also an indicator for iron the latter was precipitated with 20% solution of NaOH and separated by filtering.

The activity of exoproduct biosynthesis was studied using the radioisotope method. $NaH^{14}CO_3$ with the solution activity of 1 mCu in 1 mL was used. During the exponential growth phase the cultures were poured into 250 mL flasks. The test variants were supplemented with Mo^{6+} to an end concentration of 250 mg.l^{-1}. Then the flasks were sealed, supplemented with 5 mL of $NaH^{14}CO_3$ solution and placed in a shaker at 28°C. The incubation time was 17 hours.

Then bacteria were separated by centrifuging and their radioactivity was determined. Nucleic acids were extracted by trichloroacetic acid precipitation. Low-molecular substances were separated according to Kulaev's scheme (Kulaev *et al.*, 1961). The culture liquid was lyophilized, the total lipid fraction was extracted from the dry residue with a chloroform/methanol mixture (2:1), then low-molecular substances were extracted with 0.5 N $HClO_4$. The resultant extract was passed through a column containing DOWEX-50 resin in H-form. The total fraction of organic acids, nucleotides and sugars was obtained by washing the column with water. Amino acids were eluated with 0.5 N $HClO_4$. Nucleotides were adsorbed from the aqueous solution with activated carbon and eluated with an ethanol/ammonia/water mixture (27:2:73) (Pavlinova, 1971). Fractions corresponding to different classes of substances were diluted to the requisite volume, 0.1 mL samples were taken and sample radioactivity was measured. Liquid scintillation counter LKB 1219 Rackbeta was used to determine the radioactivity of cells and their metabolites.

RESULTS

Behaviour of Mo^{6+} and Fe^{3+} in 9K medium. Molybdenum and ferric iron are not resistant in 9K medium during *T. ferroxidans* growth and Fe^{2+} oxidation. Depending on their concentration and the amount of inoculum they are distributed in the liquid phase and the precipitate (Table 1). Sedimentation of Mo^{6+} is virtually absent with its initial level not exceeding 250 $mg.L^{-1}$. After the addition of over 500 $mg.L^{-1}$ Mo^{6+} its sedimentation starts at 2.4-2.5. After the addition of 750 $mg.L^{-1}$ Mo^{6+} and more, iron sedimentation is also observed. The behaviour of Mo^{6+} in 9K medium was taken into account in further experiments.

Effect of Mo^{6+} on T. ferrooxidans growth and Fe^{3+} oxidation. In the first series of experiments the ratio of O_2 uptake by washed *T. ferrooxidans* suspension (1.5 x 10^9 $cells.mL^{-1}$) in the presence of 25, 50 and 100 $mg.L^{-1}$ Mo^{6+} was measured. At 25 $mg.L^{-1}$ Mo^{6+} the rate of O_2 consumption by washed *T. ferrooxidans* cells was two times lower than in the molybdenum-free control (200 and 680 mg $O2.L^{-1}$ min^{-1} respectively) while at 50 $mg.L^{-1}$ and more (up to 500 $mg.L^{-1}$) the O_2 consumption was not observed with the concentration of bacteria ranging from 10^7 to 10^{11} $cells.mL^{-1}$. When bacterial not separated from the culture liquid were inoculated they were capable of developing at high Mo^{6+} concentrations. As can be seen from Fig. 1, addition of 10% of inoculum did not decrease the toxic effect of Mo^{6+} at 150 $mg.1^{-1}$. Addition of 15% of inoculum increased bacterial growth and Fe^{2+} oxidation resembled those in the control, i.e. molybdenum-free samples.

After the addition of 1000 $mg.1^{-1}$ Mo^{6+} to the medium, molybdenum content in the solution at pH 2.58 was 500 $mg.1^{-1}$. As can be seen from Fig. 2, after the addition of 15% of inoculum development of *T. ferrooxidans* was absent. After the addition of 30% of inoculum, bacteria actively oxidized Fe^{2+} at about the same rate as in the absence of Mo^{6+}.

Therefore, at some ratios of inoculum and molybdenum, *T. ferrooxidans* is capable of developing in the presence of high Mo^{6+} concentration in the medium without preadaptation.

Kinetics of Fe^{2+} oxidation and mechanism of T. ferrooxidans inhibition by molybdenum. Kinetics of Fe^{2+} oxidation, in the presence of Mo^{6+} acting as inhibitor and the mechanism of inhibition were evaluated by the 0_2 consumption rate measured in the polarograph at pH 1.5 and 2.5. The optimal conditions of the test were determined experimentally. Washed bacterial cells were suspended in iron-free 9K medium and exposed to 10 $mg.1^{-1}$ Mo^{6+} at 30°C during 10 minutes. Then the biomass with Mo^{6+} was placed into the cell and supplemented with Fe^{2+} solution. The analysis results (Table 2 and Figs. 3-4) show that molybdenum affects the rate of substrate oxidation without changing an apparent K_m value. This suggests the non-competitive type of inhibition of Fe^{2+} oxidation in *T. ferrooxidans* by molybdenum. The patterns of molybdenum affect on *T. ferrooxidans* at pH 1.5 and pH 2.5 are similar. An apparent K_m value at such pH values is $1,37 \pm 0,4$ and $1,25 \pm 0,5$ mM Fe^{2+} respectively, while that of Ki, 0.076 mM Mo^{6+}.

Effect of the supernatant. In studying the effect of the supernatant on *T. ferrooxidans* resistance to molybdenum we took into account the effect of metabolites combined with other factors (pH, Eh, Fe^{3+}, etc.). This series of experiments was performed according to the scheme of acute experiments. Cells were separated by centrifuging and transferred into fresh nutrient medium supplented with different amounts of supernatant and Mo^{6+} at 1000 $mg.1^{-1}$. After the sedimentation the molybdenum content of the medium decreased to 500-800 $mg.1^{-1}$. The analysis results are presented in Fig. 5. It can be seen that the rate of 0_2 consumption by *T. ferrooxidans* was related to the amount of supernatant added. Thus, after the addition of 30% of supernatant the rate of 0_2 consumption by *T. ferrooxidans* was the same as in the control, in the presence of 20% of supernatant it decreased while in the presence of 10% of supernatant it was equal to zero.

To elucidate the mechanism of Mo^{6+} effect on bacteria its distribution in the culture was studied (Table 3). It is noted that with the increase of the amount of inoculum the level of Mo^{6+} in the fine suspension settled at 1500xg increases while that in the centrifuge and in bacterial cells decreases.

Therefore, the toxic effect of Mo^{6+} is associated with its sorption by cells. Owing to protective properties of the culture liquid, the percent of molybdenum sorption by the cells after the addition of 20% of culture liquid is 3.3 times lower than in the control experiment without the culture liquid.

Investigation of exometabolites composition. In studying exometabolites which decrease or eliminate the toxic effect of Mo^{6+} on *T. ferrooxidans*, the radioisotope method ($NaH^{14}CO_3$) was used. As can be seen from Table 4, the highest amount of labelled carbon is incorporated in cells (66-74%). However, considerable amounts of labelled carbon are also found in exometabilites (25.5-34%). In the presence of Mo^{6+} the rate of RNA and DNA excretion into the medium increases, which is probably related to the partial lysis of cells. As can be seen from the table, molybdenum is mainly found in the fraction of low-molecular substances (94%) and partly in bacterial cells (6%). The bulk of labelled exoproducts in the medium is represented by amino acids and nucleotides (Table 5). Sugars were not detected in the medium.

Amino acids as the most actively excreted substances were studied in detail using the chromatography method. The analysis results are presented in Table 6. It can be seen that in molybdenum-free experiments the bulk of amino acids is represented by glycine, serine and aspartic acid. After the addition of Mo^{6+} the dominant amino acids are glutamic, aspartic and glycine. Valine and threonine were detected in the molybdenum-containing experiment but were absent in the control. Thus, the addition of molybdenum stimulates the synthesis of dibasic branched amino acids as well as amino acids containing additional amino and hydroxyl groups, i.e. potential complexing agents.

Study of protective properties of exometabolites. To evaluate the protective role of amino acids and nucleotides in the medium with Mo^{6+}, the method of their removal from the culture liquid was used. Amino acids were removed from the medium in the column with DOWEX-50 and nucleotides, by sorption with activated carbon. Besides, the culture liquid was passed through the column with sephadex G-25 to remove iron. Eluates were then adjusted to pH 1.7 with Na_2CO_3 or H_2SO_4 and added at 20% to fresh 9K medium containing a suspension of washed bacterial cells and 250 $mg.l^{-1}$ Mo^{6+}. The results of investigations are presented in Fig. 6. It can be seen that the protective properties of the culture liquid were retained only when nucleotides were removed. However, this does not indicate that they are not involved in the binding of some Mo^{6+}. After the removal of amino acids and ferric iron the protective properties of the culture liquid were completely lost. Apparently, the major role in the binding of Mo^{6+} is played by amino acids which form composite iron-molybdenum complexes.

CONCLUSIONS

Ions of heavy metals are known to be toxic to *T. ferrooxidans*, though in some conditions their resistance can be high. The mechanism of resistance has been insufficiently studied. However, several approaches to studying this phenomenon have already appeared. The resistance of *T. ferrooxidans* to some metals (U, Cd, Hg) is associated with plasmids (Martin *et al.*, 1983, Robinson and Tuovinen, 1984). In other cases, it can be related to enzymatic transormation of ions into less toxic compounds as, for example, in the case of mercury (Robinson and Tuovinen, 1984).

It has been reported that the oxidation of sulfide minerals by *T. ferrooxidans* is accelerated in the presence of yeasts *Rhodotorula glutinosa* (Paknikar, 1988). This effect is assumed to be related to the metal ion chelation by their metabolites. This mechanism of the decrease of metal ion toxicity is attractive since EDTA as a chelating agent is known to decrease the toxicity of U, Hg, Cd (Mahapatra a. Mishra, 1984) while addition of cysteine precludes inhibition of *T. ferrooxidans* by Ag^+ ions.

During Fe^{2+} oxidation, increased resistance of *T. ferrooxidans* to Mo^{6+} is related to its chelation and partial sedimentation in the presence of Fe^{3+} ions. In acidic medium containing Fe^{2+}, Mo^{6+} is reduced to Mo^{5+}:

$$Na2(Mo^{6+})0_4 + (Fe^{2+})SO_4 + 3H_2O \rightarrow (Mo^{5+})O(OH)_3 + Fe^{3+}(OH)_3 + Na_2SO_4.$$

During the concurrent sedimentation of colloids of Fe^{3+} and Mo^{5+} hydroxides, virtually insoluble molybdenum-containing iron hydroxide is produced (Mikhailov, 1962). At high concentrations (750-2000 mg.l^{-1}), some Mo^{6+} precipitates with iron; at lower concentrations of Mo^{6+} (250 mg.l^{-1} and less) Mo^{6+} remains in the liquid phase in the fine suspension settled by centrifugation (1500xg) and, probably, in the complex with exometabolites and Fe^{3+} ions. An especially important role in this process is played by amino acids and ferric iron, since their removal from the culture liquid results in the complete loss of its protective properties. Apparently, amino acids form composite iron-molybdenum complexes. This mechanism of *T. ferrooxidans* resistance may be also valid with respect to other metals. There is also ample evidence suggesting that adaptation of T. ferrooxidans resistance may be also valid with respect to other metals. There is also ample evidence suggesting that adaptation of *T. ferrooxidans* to heavy metal ions is nothing else but appearance of mutants with increased synthesis of chelating exometabolites.

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TABLE 1

Dynamics of Mo^{6+} and Fe^{3+} Content of the Culture Liquid

Inoculum	Mo^{6+}			Fe^{3+}		
(%)	amount added ($mg.l^{-1}$)	content in medium in 3 hours ($mg.l^{-1}$)	content in precipitate (%)	amount added with inoculum ($mg.l^{-1}$)	content in medium in 3 hours ($mg.l^{-1}$)	content in precipitate (%)
10	500	460	8.7	977	977	0
20	500	421	18.7	1675	1675	0
30	500	500	0	2443	2443	0
10	750	484	36.4	977	907	7
20	750	423	43.6	1675	1636	8
30	750	534	27.5	2443	2303	5.7
10	1000	472	52.8	977	837	15.3
20	1000	396	60.4	1675	1466	12.5
30	1000	484	51.6	2443	2164	11.5
10	1500	527	64.9	977	698	28.5
20	1500	357	76.2	1675	1186	29.2
30	1500	444	67.6	2443	1884	22.9
10	2000	572	71.4	977	698	28.5
20	2000	374	81.3	1675	1047	38.2
30	2000	443	77.8	2443	1675	31.4

TABLE 2

Effect of Mo^{6+} on O_2 uptake by washed *T. ferrooxidans* cells during Fe^{2+} oxidation (biomass concentration 2.6×10^9 cells. $m1^{-1}$; pH 2.5)

Fe^{2+} content mM	0_2 uptake rate; $mg.1^{-1}$; $min.^{-1}$			
	Mo-free		10 $mg.1^{-1}$	Mo^{6+}
	pH-2,5	pH-1,5	pH-2,5	pH-1,5
0,18	0.09	0,11	0.04	0,04
0,45	0.20	0,23	0.086	0,08
0,89	0.26	0,34	0.13	0,16
1,79	0.38	0,43	0.18	0,17
4,46	0.55	0,52	0.20	0,22
8,93	0.67	0,70	0.23	0,26
17,86	0.65	0,71	0.23	0,24
44,64	0.65	0,69	0.29	0,31
89,30	0.65	0,69	0.31	0,33
178,60	0.70	0,72	0.32	0,33

TABLE 3

Distribution of Mo^{6+} in *T. ferrooxidans* culture (Mo^{6+}, 250 $mg.1^{-1}$; duration of the experiment, 18 hours)

Experiment variants	Oxidized in 5 hrs. ($g.1^{-1}$)	Mo^{6+} content in fractions (%)			
		sediment (1500xg)	water-washed biomass	wash water	supernatant
Cell suspension in fresh medium	0	5.8	14.2	8.0	72
Same + 10% of culture liquid	2.0	13.9	9.92	5.0	71.2
Same + 20% of culture liquid	3.1	43.1	4.26	4.2	48.4

TABLE 4

Distribution of labelled products and Mo^{6+} in different fractions in *T. ferrooxidans* culture

Fraction	Distribution of ^{14}C labelled products		Distribution of Mo^{6+} in different fractions, %
	C (nmole.ml^{-1}	C (%)	
Bacteria	28.3/20.28	66.0/74	6.0
Low-molecular substances	13.16/6.8	30.7/24.81	94.0
Lipids	0.25/0.18	0.58/0.64	0
RNA	0.32/0.003	0.76/0.01	0
DNA	0.75/0.001	1.76/0.005	0

Note: numerator, 9K medium with Mo^{6+} (250 mg.1^{-1}); denominator, same without Mo^{6+}.

TABLE 5

Amount of labelled low-molecular substances in the medium (Mo^{6+}, 250 mg.1^{-1})

Substance	Distribution of ^{14}C in exoproducts	
	nmole.ml-1	%
Lipids	0.89	5.53
Nucleotides	4.37	27.0
Amino acids	6.02	37.19
Organic acids	2.66	16.44

TABLE 6

Amino acids excreted by *T. ferrooxidans* into the medium during Fe^{2+} oxidation

Amino acids	Culture without Mo^{6+} (nmole.ml^{-1}/%)	Culture with Mo^{6+} 250 mg.l^{-1} (nmole.ml^{-1}/%)
Aspartic	0,14/10.11	0,29/6.84
Threonine	none	0,13/3.16
Serine	0,27/19.5	0,20/4.64
Glutamic	none	1,34/31
Glutamine	none	0,22/4.85
Glycine	0,89/60.65	1,22/28.9
Alanine	0,12/8.83	0.10/0.20
Valine	none	0,016/0.38
Ethanolamine	0,012/0.89	0,10/0.23

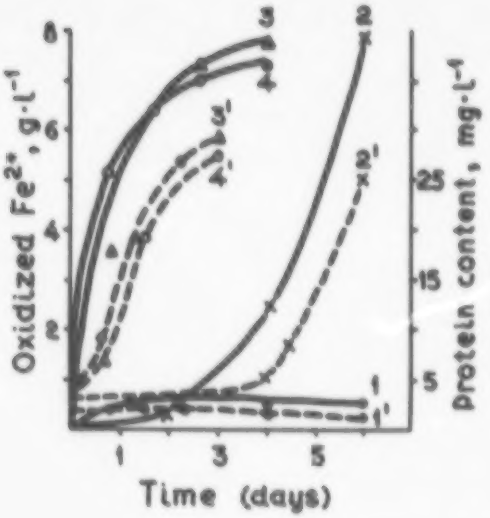

Fig. 1 Effect of inoculum amount on *T. ferrooxidans* growth (curves 1^1-4^1)and Fe^{2+} oxidation (curves 1-4). Mo^{6+} content, 150 mg.l^{-1}. Added inoculum (%): 1 – 10; 2 – 15, 3 – 20, 4 – 15 (without Mo^{6+}).

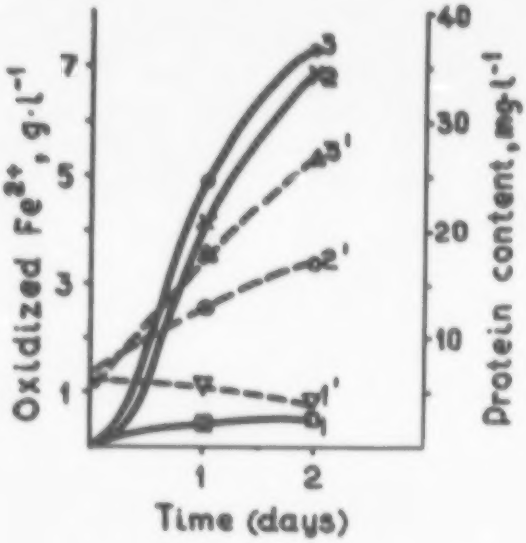

Fig. 2 *T. ferrooxidans* growth (curves 1^1-3^1) and Fe^{2+} oxidation (curves 1-3) at 500 mg.l^{-1} Mo^{6+} (added 1 g.l^{-1}). Added inoculum (%): 1 – 15, 2 – 30, 3 – 15 (without Mo^{6+}).

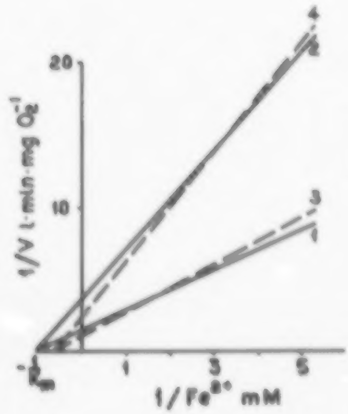

Fig. 3 Determination of the inhibition type and K$_m$ at pH 2.5 (1 and 2) and 1.5 (3 and 4) using Lineweavar-Burk method: 1 and 3 – without Mo; 2 and 4 – added 0.10 mM Mo^{6+}).

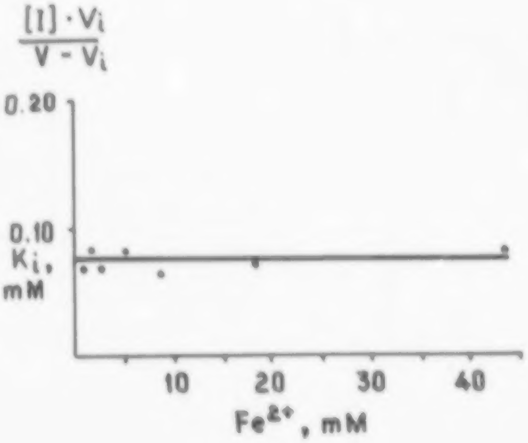

Fig. 4 Determination of the inhibition constant at pH 1.5 and 2.5 using the Hunter-Downs method (added 0.10 mM Mo^{6+}).

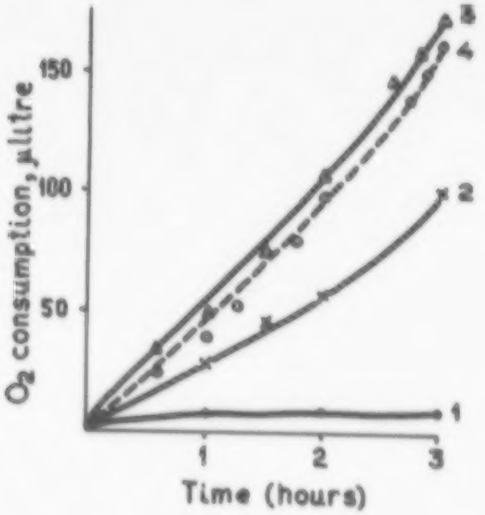

Fig. 5 O$_2$ uptake by washed *T. ferrooxidans* cells after the addition of 1 g.1^{-1} Mo^{6+} and different amounts of supernatant: 1 — 10%; 2 — 20%, 3 — 30%; 4 — 10% without Mo^{6+}. Mo^{6+} content of the medium following partial sedimentation (mg.1^{-}1): 1 — 824; 2 — 537; 3 — 550; pH 2.5-2.6; cell number 1x10^9 cells. ml^{-1}).

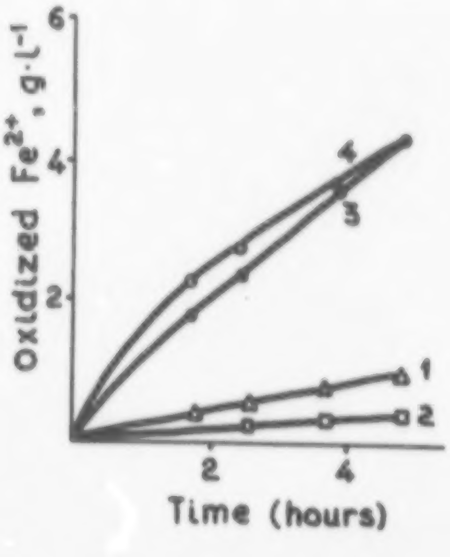

Fig. 6 Oxidation of iron by *T. ferrooxidans* in the medium containing 20% culture liquid passed through the column with sephadex G-25(1), with DOWEX-50(2) and with ativated carbon (3) 4-Mo-free control (pH-1,7, Mo^{6+}-250 mg.1^{-1}, cell number 7,6x10^9 cells. ml^{-1}).

CASE AND PILOT STUDIES – LEACHING

BIOLEACHING OF URANIUM AT DENISON MINES

R.G.L. McCready and W.D. Gould

CANMET
ENERGY, MINES AND RESOURCES, CANADA 555 Booth Street, Ottawa, Ontario K1A 0G1

ABSTRACT

Over the past four years Denison Mines, Elliot Lake, Ontario, in co-operation with CANMET and three Canadian Universities have developed a commercial process for the bacterial leaching of low grade uranium ores. In 1984, Denison Mines signed an agreement with the National Research Council, Industrial Research Assistance Program to determine the feasibility of utilizing bacterial leaching for commercial production of uranium. Laboratory studies on the microbiology of the process were initiated at Dalhousie and Laurentian Universities and Denison conducted studies on the drilling and blasting patterns required for ore breakage. At the completion of the research and development program Denison Mines continued modification of the process and currently produce 10-15% of their total production by bacterial leaching.

LA BIOLIXIVIATION DE L'URANIUM AUX MINES DENISON

R.G.L. McCready et W.D. Gould

CANMET

Énergie, Mines et Ressources Canada 555, rue Booth Ottawa (Ontario) K1A 0G1

RÉSUMÉ

Les mines Denison, Elliot Lake, Ontario collaborent depuis quatre ans avec CANMET et trois universités canadiennes pour mettre au point un procédé commercial de biolixiviation pour des minerais à faible teneur en uranium. En 1984, les mines Denison ont signé une entente avec le Conseil National de Recherche dans le cadre du programme d'assistance en recherche industrielle afin d'évaluer si la biolixiviation de l'uranium pouvait être exploitée de façon commerciale. En laboratoire des études microbiologiques ont été entreprises aux universités Dalhousie et Laurentienne, et les mines Denison ont effectué des travaux sur des méthodes pour forer et rompre le minerai. Lorsque le programme de recherche et développement s'est achevé, les mines Denison ont continué à modifier le procédé et présentement la lixiviation bactérienne compte pour 10-15% de leur production totale d'uranium.

INTRODUCTION:

Heap leaching of low grade ores to recover metal values was first employed in Germany in the 1500's; however, the role of the acidophilic Thiobacilli in metal leaching was not realized until their isolation from acidic mine drainage by Colmer and Hinkle in 1947. The commercial application of heap leaching for the recovery of uranium from low-grade ores or mine wastes in North America was first reported by Mashbir (1964).

In the early 1960's, mine operators at several of the uranium mines in the Elliot Lake area in Northern Ontario noticed that the mine drainage had become very acidic and contained large quantities of soluble iron and uranium (Harrison *et al.*, 1966). Harrison and his co-workers isolated *Thiobacillus ferrooxidans* from Denison mine water and showed that the bacteria were required for the extraction of uranium from the ore. In the early 1970's all but two of the eight mines in the Elliot Lake area had shut down. Economic quantities of uranium were recovered from these mines however, by periodically spraying the stope walls with acidic drainage (Fisher, 1966; MacGregor, 1969; Fletcher, 1970). In 1964 and 1965 a total of 68,300 kg of U_3O_8 were recovered by this spraying technique from Rio Algom's Milliken Mine which had suspended mining operations (Fisher, 1966).

In most ores the uranium occurs as a mixture of minerals containing the uranium in either the tetravalent or the hexavalent state. Uranium is soluble only in its most oxidized hexavalent state (Brierley, 1978; Lundgren and Silver, 1980). Tetravalent uranium can be oxidized to the hexavalent state by ferric iron, but the oxidation occurs much more rapidly in the presence of the iron-oxidizing *Thiobacillus ferrooxidans* (Lundgren and Silver, 1980).

$$UO_2 + Fe_2(SO_4)_3 \longrightarrow UO_2SO_4 + 2FeSO_4$$

Bacterial leaching of uranium occurs via an indirect mechanism, in which the bacteria oxidize the pyrite within the ore, generating an acidic ferric sulphate solution which carries out the chemical oxidation of the tetravalent uranium to the soluble hexavalent state.

Attempts have been made to optimize the bacterial leaching of uranium during the stope spraying operations in the various Elliot Lake mines. Duncan and Bruynesteyn (1971) reported that addition of phosphate to the wash water had no effect on the leaching rate for uranium, but that increased rates were observed on the addition of ammonium ion or ferrous iron. In their studies, ammonium sulphate was added at a rate of 1.4kg/ stope, potassium phosphate 0.23 kg/stope and ferrous sulphate 11.4 kg/ stope as dry powder or sprayed over the stope as a concentrated solution. As various volumes of water were used, and the sizes of the stopes and the quantity and grade of the muck varied, valid comparisons of the uranium leaching rates under the different experimental conditions could not be determined.

BIOLEACHING OF URANIUM AT DENISON MINES, ELLIOT LAKE, ONTARIO

In 1984, Denison Mines, with support from CANMET, negotiated a cost-shared agreement under the National Research Council Biotechnology Industrial Research Assistance Program (IRAP) to develop a bioleaching process to commercial scale for the recovery of uranium.

The spatial geometry of the ore body in the Denison Mine is conducive to bacterial leaching. The lower reef is separated from a lower grade upper reef by about 2 m of barren rock. As the lower reef has been mined by conventional room-and-pillar mining and the uranium has been recovered by sulphuric acid leaching, ion exchange and ammonium precipitation, the upper reef may be drilled and blasted into these large stopes, which are ideal leaching vessels.

The Denison orebody is on the Quirke Lake syncline and makes up a part of the Huronian sediments in the region. The uranium bearing minerals, brannerite, monazite, and uraninite are concentrated at the pebble/quartz interfaces in this quartz pebble conglomerate. The ore has a specific gravity of 2.75, is in excess of 75% silica and contains approximately 7% pyrite-pyrrhotite. The upper reef has an average grade of about 0.4 kg /tonne (Wadden and Gallant, 1985).

The project had several research objectives as outlined below:

- to determine the drilling pattern required to provide optimal ore fragmentation
- to determine whether or not bacterial nutrient supplementation was required for optimal leaching.
- to attempt to isolate, identify and test psychrophilic strains of *T. ferrooxidans*
- to compare spray-trickle leaching to flood leaching.
- to develop a method of protecting workers from the higher levels of radon emanations resulting from the large volumes of fragmented ore.
- to develop an aeration system capable of supplying sufficient air to maintain the growth of *T. ferrooxidans*.

DRILLING PATTERN TESTS:

During the initial phases of the study Denison utilized three different drilling patterns in their test program. The base patterns were a standard 0.91m x 1.22m spacing which had been utilized by Denison for some time; 0.91m x 0.91m; and a 0.61m x 1.22m spacing of drill holes.

Two different types of drill rigs were used to drill the 60,000 m of holes required for the tests; electric hydraulic uppers jumbos and air-operated bar and arm drills. Once drilling was completed, perforated air lines were installed on the floor of the stope and carefully covered with ore to prevent their collapse during blasting. Once the compressed air lines were in place and protected, the stopes were blasted and photographically analyzed by the Canadian Industries Limited (CIL) Blasting Physics Section.

Their report stated that the 0.91 x 0.91 m drilling pattern produced adequate results provided the proper blasting initiation pattern was adopted. The closer drilling patterns appeared to produce only marginally better results, based on photographic analysis.

BACTERIAL NUTRIENT OPTIMIZATION STUDIES:

Concurrent with the Denison Mines research on stope development, Dalhousie University, under contract to Denison, was investigating the nutrient composition of the acidic mine drainage and isolating indigenous species of *T. ferrooxidans* from various locations within the mine. From both shake flask leaching tests and laboratory column leaching tests, it was concluded that there was sufficient nitrogen (from the explosives), magnesium and iron (from ore constituents) to support growth of the thiobacilli. The mine water, however, was deficient in phosphate (Table I). A recommendation was made to Denison that sufficient H_3PO_4 be added to their mine water for stope leaching to produce a phosphate concentration of 15-20 ppm (McCready *et al.*, 1986). *T. ferrooxidans* species were isolated from six different locations within the mine; however, during tests on the effect of temperature only two of the isolates would grow below 15°C. When the sump locations were checked, it was noted that the two cold-tolerant strains had been isolated from sumps which were within 50 m of a mine ventilation intake shaft. Similar findings were reported by Laurentian University from their contract work which involved the isolation of psychrophilic strains of *T. ferrooxidans* (Ferroni *et al.*, 1986). Thus, during the cold winter months these two sumps would be exposed to fresh air being drawn from surface. It was recommended that one or both of these sumps be used as inocula for the in-situ stope leaching as the organisms associated with these sumps were somewhat psychrophilic.

UNDERGROUND BACTERIAL COLUMN LEACHING STUDIES:

Six large columns were constructed and installed in the underground research laboratory at the Denison Mine. The columns were 3 m high and 0.61 m in diameter, constructed of rubberized mild steel with open tops, and each column had a capacity for just under 1.5 tonnes of broken ore. Tests were carried out to determine the effect of phosphate supplementation of the mine water and to assess the effect of inoculation with a large volume of laboratory-grown cells from Laurentian University.

When the data from these two test columns were compared to the leaching data obtained from two columns leached with indigenous mine water, large-scale inoculation resulted in a 15% higher uranium extraction than was observed in the control column. The phosphate-supplemented column showed a 12% greater extraction than that of the control column. However, the large bacterial inoculum was grown on 9K medium, and the leach solution within this column had as much residual phosphate as the nutrient-supplemented column.

LEACHING OF URANIUM AT THE DENISON MINE:

For the stopes selected for flood leaching, the excess ore was removed from the stope after blasting and a large hitch was cut across the floor of the stope, up both sides of the entrance and across the top (ceiling). A reinforced concrete bulkhead, capable of supporting the head pressure of up to 48 psi, was constructed in each stope.

Once the concrete bulkheads had cured, the flood leaching cycle was started. Acid mine drainage, which generally was at pH 2.3 and had a redox potential of >450 mV, was pumped into the stope to completely cover the ore. Flooding required three days of continuous pumping at a rate of 25 L/second and then an additional three days were required to drain the stope. Once drained the stope was allowed to react for a three week period and then the flooding cycle was reinitiated. After the first reaction period the uranium liquor drained from the stope during the second flooding is pumped to the surface mill for recovery by ion exchange followed by ammonium precipitation to form yellowcake (Campbell *et al.*, 1987).

A cost comparison was made by Denison Mines between their flood leaching and their spray leaching processes. Mining costs for the two techniques are equivalent, construction costs are slightly higher for flood leaching, but roof bolting, ventilation and maintenance costs are much greater for spray leaching. Considering all the costs, the flood leaching technique is less expensive per tonne of ore treated than is the spray leaching technique. Also, from a safety point of view, the flood leaching technique is much safer as the broken ore is contained in a "sealed" container and stope ventilation is more easily controlled to remove the radon gas emanating from the large volumes of broken ore. In contrast, the spray leaching stopes are open, require roof bolting and a high ventilation rate is required to allow the maintenance crews access to the stope to service the sprinkler systems.

RESULTS OF THE DEVELOPMENT PROGRAM:

During 1987 Denison Mine personnel mined through into each of the initial six stopes to obtain tails samples to determine the degree of ore fragmentation and analyze for the uranium in the residues (Table II, Table III).

From the data in Tables II and III it is apparent that the grade of the ore was highest in the smaller size particles. Except for the larger size fractions the leached ore fragments (Table III) show much lower grades than the head sample (Table II). Also, leaching tends to increase the percentage of finer material due to the removal of the uranium mineralization as well as the pyrite; which allows disintegration of the particles. Similar material breakdown has been observed during the bacterial leaching of pyrite from coal (McCready, 1985; McCready and Zentilli, 1985). From the test stopes the overall recovery of uranium ranged from 69-86% of the available metal values.

EFFECT OF TEMPERATURE:

During the winter the acid mine drainage within the Denison Mine has an average temperature of 12°C and the air temperature will vary from 0 to 15°C. During the late summer months the air temperature underground will range as high as 22°C but the high humidity within the mine causes "sweating" of the rock faces. Although the ambient temperature is elevated the temperature of the mine drainage is consistently around 12°C (McCready, 1988).

Mine personnel can easily determine if a particular stope is actively leaching. In the early stages of the project it was noted that although the 1.6 million gallons of acid mine drainage being pumped into the stope were at 12°C, when the stope was drained the solution temperature was often as high as 15°C.

Seasonal variation in the air temperature greatly affects the bacterial leaching process. During the late summer, when the air temperature and humidity within the mine are high, the rate of uranium extraction is about three times higher than during the winter.

PRODUCTION LEVELS AT DENISON:

The mine plan for 1988 projected that 1,100,000 tons of ore would be maintained as an inventory for bacterial leaching and 90 flood leaching stopes were in various stages of operation or in preparation for flood leaching. During 1987 the Denison mine produced about 452,000 kg of uranium from their leaching operations. The forecast for 1988 was to increase production to 540,000 kg of uranium. However, due to mining problems in a particularly steeply sloped area of the mine, only 347,000 kg of uranium were produced from the leaching operation in 1988.

RECENT MODIFICATIONS IN THE DENISON LEACHING PROCESS:

Originally, Denison personnel would lay perforated pipes on the floor of the stopes and during the leaching process, compressed air was pumped through these pipes to provide air for the microorganisms. As the number of flood leaching stopes increased, the compressed air requirements became prohibitive; thereby affecting production in the conventional mining areas. Denison conducted a study in which high pressure blowers (fans) were installed at the top of the stopes and connected through the top bulkhead to two-inch diameter perforated plastic pipes that were laid on the stope floor prior to the blasting of the stope. During the rest cycle these fans blow air through the muck pile supplying air for the leaching process. Over-pressuring of the stopes is avoided by venting through holes drilled in the highest part of the stope. This procedure not only provides aeration of the muck pile but removes the radon gas from the stope and it is eventually vented to the atmosphere via an independent exhaust duct network (Marchbank, 1987).

Labour and construction costs for the leaching process have steadily declined over the past few years at Denison. Through the experience gained in the construction of the large number of bulkheads the crews have become more efficient and labour costs have been greatly reduced. Also, as the leaching personnel gain more experience they have varied the flood/drain cycles to improve the grade of the pregnant liquor; thus reducing the volume of low-grade liquor being pumped to surface.

A substantial quantity of the ore in the Denison reserves has been affected by intrusive diabase dykes which were formed after ore deposition; this ore cannot be treated by conventional extraction techniques. A large sample of freshly-broken, chloritic ore was divided into two parts, one part was left as mined and the second part was crushed to -51mm. Each sample was loaded into a 0.61m x 3m leaching column in the underground laboratory and flood leached on a monthly cycle.

As the chloritic ore contains apatite, the addition of phosphate to the leach solution was not necessary. Leaching of the chloritic ore produces high iron concentrations (6g/L) during the drain cycle; the iron concentration of the interstitial water in the muck pile may be as high as 20g/L, which may promote ferric hydroxysulfate or jarosite precipitation within the pile.

The leach residues from these two columns were collected and analyzed and 67% extraction was achieved with the run-of-mine sample and 77% extraction was achieved with the crushed chloritic ore sample. Therefore, uranium may be economically recovered from this refractory ore.

EFFECT OF LEACHING ON DENISON OPERATIONS:

The success of the bacterial leaching process has brought on stream an additional 4,000,000 tonnes of chloritic ore which could not have been processed by conventional technologies. Tens of millions of pounds of uranium will be recovered from low grade ore without the production of tailings which must be stored on the surface. As the leached ore remains underground there are no environmental consequences due to the mining and uranium extraction. Although exact processing costs figures are not available, Denison estimates that the cost per pound of uranium is substantially lower than their costs for the conventional sulphuric acid extraction. Bacterial leaching allows recovering of mineral values from the low-grade ore and the recovery of values from the normally refractory chloritic ore.

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Table I

Major ion contents of Denison Mine water samples in ppm (mg/L)

Sample	$H_2PO_4^-$	Mg^{2+}	NH_4^+	NO_2^-	NO_3^-
30D + 32N discharge	3	15	50	7	170
46076 discharge	4	10	50	7	190
46078 discharge	9	12	75	11	350
26305 sump discharge	1	8	20	2	95
31881 sump discharge	3	7	50	6	162
Total mine water	1	15	30	3	170
Average values	3.5	11.2	45.8	6	189.5

Table II

Particle size analyses and uranium content of fragmented ore prior to leaching.

Size Fraction	% Distribution	Grade (lb/ton)
>12 inches	18.26	0.47
>8 <12	14.37	0.23
>6 <8	9.67	0.42
>4 <6	13.44	0.35
>2 <4	21.79	0.61
>1 <2	11.05	0.55
>0.5 <1	3.83	0.32
>0.25 <0.5	1.94	0.81
<0.25	5.66	1.11

Table III

Particle size distribution and grade of the ore from the test stopes after bacterial leaching.

Fraction Size	Average % Distribution	% Distribution Range	Grade (lb/ton)
> 12 inches	13.97	5.9 - 22.4	0.23
>8 <12	7.11	3.4 - 13.7	0.39
>6 <8	6.55	2.7 - 10.8	0.36
>4 <6	7.25	3.1 - 11.5	0.32
>2 <4	16.04	11.7 - 19.8	0.26
>1 <2	13.32	8.7 - 15.5	0.21
>0.5 < 1	10.71	5.9 - 14.0	0.16
>0.25 <0.5	8.12	7.5 - 10.6	0.13
<0.25	16.93	7.2 - 23.8	0.18

IDENTIFYING THE BIOHYDROMETALLURGICAL PROCESSES WITH THE GREATEST PROBABILITY OF COMMERCIAL ADOPTION

Keith H. Debus

Department of Mineral Economics
Montana College of Mineral Science and Technology

ABSTRACT

After many years of research, several biohydrometallurgical processes have achieved or are approaching commercial application. When these processes are viewed from a process economics perspective, it becomes clear that they possess some common attributes that have been critical to their commercial success. These attributes, when overlaid with an astute analysis of the economic bottlenecks of the minerals industry, can provide valuable insights into the areas where biohydrometallurgy is likely to make its future inroads. These insights may then enable the private sector, government, and academia to focus their limited time and resources into research in these areas with the greatest probability of commercial adoption and success.

This paper will identify: first, the common characteristics of those processes that have achieved or are approaching commercial adoption; and second, the economic bottlenecks of the minerals industry as identified by a survey of industry leaders and the author. From the intersection of these two sets of parameters, the biohydrometallurgical processes with the greatest probability of commercial adoption and success will be identified.

DÉTERMINATION DES PROCÉDÉS BIOHYDROMÉTALLURGIQUES PRÉSENTANT LES MEILLEURES CARACTÉRISTIQUES POUR L'UTILISATION COMMERCIALE

K.H. Debus

Département d'Économie minérale
Collège des Science Minérale et de la Technologie du Montana, É.-U.

RÉSUMÉ

Après de nombreuses années de recherches, plusieurs procédés biohydrométallurgiques sont utilisés commercialement ou sont sur le point de l'être. Quand ces procédés sont évalués du point de vue économique, il apparaît évident qu'ils présentent des qualités communes dont l'importance a été déterminante dans le cadre de leur utilisation commerciale. Ces qualités, mises en relation avec une analyse poussée des restrictions économiques de l'industrie minière, permettent d'obtenir des renseignements fort utiles sur les secteurs dans lesquels la biohydrométallurgie est appelée à faire des percées. Ces renseignements permettent ensuite aux entreprises privées, au gouvernement et aux institutions de recherche de concentrer leur temps et leurs ressources limités sur des secteurs de recherche présentant les meilleures perspectives en matière de commercialisation et de rentabilité.

Le présent document indique d'abord les caractéristiques communes des procédés qui sont utilisés commercialement ou qui sont sur le point de l'être et, dans un deuxième temps, il mentionne les restrictions économiques auxquelles se heurte l'industrie minière; ces restrictions ont été indiquées par les dirigeants des principales industries, ainsi que par l'auteur. À partir de ces deux groupes de données, il est possible de définir quels sont les procédés biohydrométallurgiques les plus susceptibles de connaître une utilisation commerciale.

INTRODUCTION

Just as biohydrometallurgy's preceding three decades have been marked by a focus upon the scientific and engineering aspects of the iron-oxidizing bacteria *Thiobacillus ferrooxidans*, the next decade will likely be marked by the rapid and widespread commercial developments in biomining. Biohydrometallurgy has held significant promise for the mining industry for those three decades, but only now has the necessary critical mass congealed to make this long-anticipated leap to widespread commercial adoption. This critical mass includes sufficient commercial success to have converted advocates among the mining industry and most recently, the confidence of the financial community that it holds the promise of significant profits. The next decade then will be one of revolutionary change in the mining industry, as resource recovery techniques are likely to change more in the next ten years than in the past millennium. For this reason, it is necessary now to step back and examine the future of these biological processes and the resource recovery industry. In this way, we may be able to avoid several technological dead ends and focus the industry's efforts on those areas that are likely to pay the greatest dividends.

In an effort to identify those biological processes that will be commercially viable for the resource recovery industry, some intellectual groundwork must first be laid. First, note that I have avoided using the term "mining" industry when I discuss the future. This is intentional and is a reflection of the magnitude of the change biotechnology will have on this industry. In this new environment of metal and mineral recovery, the term mining will seem anachronistic. Contemporary mineral and metal recovery techniques are more similar than dissimilar with those mining technologies utilized over the last three thousand years. This industry has made enhancements in the level of sophistication to the technology that was initially used in the ancient world, but has failed to transform this technology significantly. For purposes of contrast, during the same period of time, both health care and information processing technology have revolutionized their functions in industrial society. It does not require a historian of technology to comprehend that health care and information processing technologies have radically transformed not only their tools, but also the way we think and understand these functions of industrial society. Unfortunately, the same cannot be said for mining technology. Biotechnology will provide this industry with an entirely different conceptual paradigm of mineral or resource recovery as these ancient techniques and technology become obsolete. Thus, I prefer the term "resource recovery" to signify the industry's post-biotechnology advent and adoption.

Second, as Robert Noyce (1989), founder of Intel, and now CEO of Sematech (the semiconductor industry's joint research and development effort), has so concisely stated:

> A lot of things are technologically possible, but only economically feasible products will become a reality. Where costs can be pushed down rapidly, great new vistas arise.

This statement is as pertinent to biotechnology in the minerals industry as it is to the semiconductor industry. Technological developments in themselves make for excitement in the laboratory and in academic journals, but only those that can contract costs profoundly will have the impact on industry and society that this analysis is attempting to identify. Many biotechnology processes will or have become technologically feasible, but history indicates that it is not strictly a matter of simple economics, but rather of technology and techniques that can compress costs sharply and open "great new vistas" that transform industries and society. It is techniques and processes capable of opening these new vistas that this analysis seeks to identify.

Third, this analysis is attempting to identify only those processes that will survive the long-term in the industry. In other words, those techniques or processes that may gain some economic advantage in the short-term — but lead to technological or commercial dead ends — will be filtered out. As Herbert A. Simon (1977), the Nobel Laureate economist and cognitive scientist so aptly pointed out:

> Prediction is easier (and presumably more accurate) if we do not try to forecast in detail the time path of events and the exact dates on which particular developments are going to occur, but to focus, instead, upon the steady state that the system is tending.

An example from history of such analysis is provided by computer technology. The computer was invented in 1840 by Charles Babbage (Mergerison,1978) and although many technological improvements were made upon his machine over the next century (each having economic advantages over the previous system), none had the effect of sharply and significantly reducing the cost of computing. This was because none of these technological enhancements addressed the primary economic bottleneck of computing, that is, the limitations of analog systems. As a result, to have projected into the future these analog devices would have missed the steady state that this system was tending. It took the insight of Allan Turing in 1936 to identify this barrier and suggest digital systems as the solution. Turing's analysis in 1936 then is responsible for having made these machines a ubiquitous part of our modern world as computing technology approaches its steady state. This analysis attempts to identify the steady state for the resource recovery industry by identifying those biological resource recovery processes that address the needs of the industry in a manner that will dramatically reduce costs.

In order to properly and accurately identify those processes that will shape the future of the resource recovery industry, it becomes incumbent to undertake two (2) key tasks. First, we must identify where the mining industry's problems and economic "bottlenecks" occur, as Turing did with the computer. These are the production or process areas that have resisted significant cost reductions by the application of new technology or where costs have increased as a result of changing conditions. It is here that new technology can have its most significant impact. Second, we must identify those characteristics of biohydrometallurgical processes that will provide us with the opportunity to reduce costs of resource recovery sharply in order to reveal these "great new vistas" that Mr. Noyce refers to. It is the intersection of these two sets — those biological processes or process characteristics that address directly the industry's greatest barriers to significant cost reduction — that is likely to unveil the identity of the successful commercial processes of the steady state resource recovery industry.

ECONOMIC PROBLEMS AND "BOTTLENECKS" FACING THE MINING INDUSTRY

First let's address the issue of economic bottlenecks. Although it has been well-documented, it is necessary here to restate the changing nature of the metals industry in the U.S. and the world. Undoubtedly, new mineral deposits will be found that are relatively high grade in the ensuing decades, but these will be the exception and not the rule. From a global and national perspective, ore grade will continue to decline as high grade ores are depleted. Since the turn of the century, the average ore grade for copper in the U.S. has declined from approximately 2% (U.S. Geological Survey, 1914) to the present .6% (U.S. Bureau of Mines, 1988). Similar patterns can be found in other base and precious metals as the high grade ores are depleted. This slow, but steady decline in ore grade will make more and more deposits uneconomical to mine by conventional means in the near future. Simply put, these lower grade ores lead to higher production costs because the firm incurs the expense of handling more material to produce an equivalent amount of metal. In the copper industry, over 50% of the energy consumed and 2/3 of the capital costs are associated with the removal of the ore and beneficiation by conventional processes (Agarwal *et al.*, 1980). With high grade ores, the metal values in the ore exceed these costs but as ore grade declines, there becomes a crossover point where the ore extraction and beneficiation costs exceed the value of the metal. See Figure 1. At this point, conventional mining techniques are no longer effective and lower cost processes become necessary. The industry may be driven beyond this crossover point by (1) a reduction in ore grade, (2) lower metal prices, (3) higher input prices, or any combination of the above.

Notwithstanding the recent attempts by the mining industry to reduce energy consumption, the industry remains relatively energy intensive. For instance, the production of a metric ton of copper by conventional mining techniques requires approximately 100 BTU's of energy of which 1/3 to 1/2 are associated with ore extraction and beneficiation (U.S. Bureau of Mines, 1988). Despite the relative stabilization of energy costs, the mining industry must be wary of a sudden and sharp rise in the cost of energy in the future. Any sharp increase in energy prices would severely inhibit the industry's ability to sustain their current prosperity. The industry must not be lulled to sleep by the pronouncements of OPEC's demise and the energy glut, as neither of the energy crises of the 1970's were strictly a result of supply and demand, but rather Middle Eastern politics. Considering the continuous political instability of the Middle East, any somnolence in the effort to reduce energy consumption by the industry could be met with an abrupt arousal and a sea of red ink.

Concern and financial liability associated with environmental damage from mining activities seems to be following an inexorable path. This trend seems to have accelerated in the preceding year, although the environmental conscience of the mining industry had been raised several times in the last decade by a number of legal and regulatory changes. Most significant among these was the limitation on sulfur dioxide emissions from smelters. This measure alone cost the copper industry over $2 billion (MacDonnell, 1987) for air pollution control and 10 to 15 cents per pound of copper produced (Everest, 1985). If the EPA holds mining companies financially liable for the clean-up costs of abandoned mining sites that have achieved Superfund site status and this financial culpability becomes more of a certainty, environmentally superior techniques will swiftly become economically superior techniques.

Labor costs of the mining industry must also be addressed. Although the industry has been successful in reducing labor costs and raising labor productivity in recent years, if several recent demographic and economic studies are correct, labor costs will once again begin rising sharply in 1990's as a result of an expected labor shortage. This anticipated labor shortage in the U.S. is the result of the large number of "depression babies" leaving the labor force as they reach retirement age combined with the effects of the "baby bust" of the 1960's and 1970's. These demographic trends will combine to produce fewer new members of the labor force in the 1990's and beyond. Just as with any commodity, if demand continues to grow while supply remains tight, the price rises. This labor shortage is expected to occur most acutely among the skilled segment of the labor force.

Academicians are often accused of talking among themselves and disregarding the fact that a real world exists beyond our ivory-covered walls. The result is often academic analyses that are sterile and have little relevance to what is actually happening in the field. In order to thwart this type of intellectual incest and augment our analysis of the mining industry, we at Montana College of Mineral Science and Technology conducted a survey of the two hundred (200) major mining companies in North America (Debus, 1988). This survey was conducted to acquire mining executives industrial perspective on some of these key issues. In an attempt to receive responses from that key company official with the broadest perspective of the industry, the survey was directed to the chief executive officer (CEO) of these firms. Assuming that these CEOs know and understand their industry, then several of their responses to this survey have relevance here.

Critical and germane to this analysis of economic bottlenecks of the mining industry, the CEOs were asked to identify that segment of their production process least competitive with foreign producers. The responses gave us a clear picture of how these CEOs view the relative competitiveness of the various production segments of the industry. Over a third of the respondents (37%) indicated that smelting and refining was the least cost competitive of their production processes, followed closely by 28% who responded that ore extraction was least cost competitive. No other segment of the mining industry production process was viewed as the least competitive by more than 10% of the chief executive officers. This data suggests that the majority of the individuals who operate North America's major minerals companies, view smelting and refining and ore extraction as their greatest economic bottleneck. These two areas that have hindered the industry's efforts to reduce costs then may be perceived as providing the greatest opportunity for biohydrometallurgical processes. Processes that address these areas cost-effectively are more likely to gain commercial acceptance as they confront the perceived barriers identified by the industry leaders. It is worth noting here that the biological mining process with the greatest commercial success to date, copper leaching, has addressed both of these issues and hence has been able to reduce copper production costs by half and gain widespread acceptance. Such a lesson should not be lost to us in our future planning and development by the biohydrometallurgy community.

In summary, ore grade continues to decline and the mining industry remains relatively energy intensive, spending large sums to transport waste rock and process ores. The industry is also likely to experience accelerating labor costs over the next decade. Environmental regulations are expected to continue to become more restrictive and therefore increase mitigation costs, while the industry leaders believe that smelting and refining and ore extraction provide the greatest barriers to reducing production costs.

KEY ECONOMIC CHARACTERISTICS OF BIOHYDROMETALLURGICAL PROCESSES

A brief review of the present state of commercial biotechnology in the resource recovery industry is necessary here. In terms of dollar value, the recovery of copper from dumps, heaps, and *in-situ* provides the overwhelming success story to this field. In 1989, over 25% of all copper produced in the U.S. will come from biological processes, or over $650,000,000 (Holmes *et al.* 1988). This copper recovery from biological means can be attributed to the work of the ubiquitous bacteria *Thiobacillus ferrooxidans*. First exploited commercially in 1752 at Rio Tinto and first identified by Bryner et al. in 1954, this organism continues to generate the bulk of the commercial and research interest in this area. These include the exciting new commercial developments in oxidizing refractory gold ore, desulfurizing coal, and recovery of uranium. In short, up to the present this bacteria has been the "workhorse" in commercial biological resource recovery despite the fact that we still understand so little about its interaction with pyrite. This means that much of the commercial research and development in this field has been engineering systems to optimize the iron-oxidizing capability of this organism.

Other processes that have gained at least some small level of commercial success include the biosorption of metals from aqueous solutions and the biological destruction of cyanide. This is not to downgrade or ignore a whole host of other biological processes for resource recovery, but they simply have not yet gained commercial adoption or acceptance to any substantial or sustained degree.

The second task necessary to complete this analysis requires that a discerning inventory of the key attributes or characteristics of the commercially successful biohydrometallurgical processes be taken. The fundamental criterion is to identify the characteristic primarily responsible for the reduction in costs or improved capability that can be credited for the success of the process. An analysis of the economics of these processes reveals the following key attributes were critical to their commercial success:

1. *in-situ* capabilities
2. lower energy costs
3. lower capital costs
4. lower labor costs
5. ability to convert waste product to a resource
6. "passive" system process
7. new capabilities not available with other technology
8. favorable environmental impact
9. less severe operating conditions (temperature and pressure)

These attributes were distributed among the commercially successful biohydrometallurgical processes as indicated in Table 1.

It is crucial here to examine in detail why these processes have been commercially successful. Beginning with copper bioleaching, it has often been reported that copper bioleaching has gained commercial favor because it is less expensive. This is undoubtedly true and succinct but such simplistic analysis does little to enlighten this analysis. Copper bioleaching's cost effectiveness is directly related to its ability to forego much of the labor, capital, and energy costs necessary in the mine development to concentration steps in the heap, dump and *in-situ* leaching setting (some of these energy savings are offset by additional energy costs in the electrowinning recovery process). Furthermore, it eliminates the environmental hazards and liabilities (sulfur dioxide and arsenic laden air effluent) inherent in smelting sulfide ores.

As for gold ore oxidation, the reasons are less clear. Once again the simple answer is that it is less expensive. Unfortunately, the answer is more complex than that. First, the capital costs of such a plant are 15-35% less costly than a pressure oxidation or roasting plant (Holmes *et al.*, 1988). Second, the operating costs, due to less severe operating conditions than the other two oxidation process alternatives, require fewer and less highly skilled operators. Furthermore, the process is not as energy intensive as the alternatives, further contributing to the lower operating costs. Finally, the biological oxidation process produces little or no air effluent (but about equal amounts of solid effluent) reducing the costs of air effluent mitigation.

The biosorption processes at this point largely have achieved their limited commercial success because of two primary attributes; (1) their ability to trap and remove metals in very low concentrations, and (2) their ability to recycle waste metals back to industry. Of these biosorption processes, those that utilize the ability of the cell envelope or other extracellular polymers to trap metals seem to carry the greatest potential for commercial development. These polymers do not require that the system be living, avoiding the additional costs associated with maintaining a living system, while being impervious to changes in metal concentration, pH, temperature, etc., that living systems are likely to find uninhabitable.

Finally, the success that biological cyanide destruction has achieved has been a result of increasingly restrictive environmental protection regulations and the ability of this process to singularly meet these regulations where the alternatives could not. Also contributing to its success has been the relative "passivity" of the process. It requires little in the way of operating costs, requiring no continual addition of reagents, only small amounts of electricity, and relatively small amounts of labor to maintain operation.

PERCEPTIONS OF TECHNOLOGICAL SOLUTIONS BY THE INDUSTRY LEADERS

Further insights for this analysis may be gained from responses to questions regarding potential technological solutions to the industry's economic problems that were included in the survey referenced above. The mining industry CEOs were asked to identify the key characteristic of a technology that is most likely to improve their competitiveness with foreign producers. Given a wide choice of possible characteristics such as less energy intensive, lower capital costs, environmentally benign, etc., they overwhelmingly identified higher labor productivity as the most important characteristic of an effective new technology to improve their competitiveness.

Finally, these CEOs were asked to identify the technology area with the greatest potential to improve their relative competitiveness. Although they failed to reach anything resembling a consensus on this issue, it is important to examine their principal selections. Both *in-situ* mining and optimization and control were the first choice of 20% of the respondents, while advanced rockbreaking and intelligent mining systems were chosen by 15%. Advanced biological recovery techniques garnered first place in 12% of the surveys (over 50% of the precious metal respondents). These responses, while less unified than the responses to the previous two questions, do display some important priorities of these leaders of the industry, most particularly their emphasis upon *in-situ* mining and advanced rockbreaking techniques.

CONCLUSIONS

Then, through the intersection of this analysis and evidence of the economic problems and bottlenecks of the mining industry with the key attributes of biohydrometallurgical processes and the insights of the mining industry leaders, we can now draw some conclusions regarding the future of biohydrometallurgy. This intersection will unveil those biohydrometallurgical processes that have the greatest probability of reducing production costs dramatically and opening the "great new vistas" described by Mr. Noyce and defining the technology and the new industry when they settle into the "steady state" that Dr. Simon referred to.

First, the iron- and sulfur-oxidizing bacteria *Thiobacillus ferrooxidans* will continue to play a pivotal role in the recovery of some metals where pyrite is present. These will continue to be copper and uranium, but will also include other sulfide metal ores that contain nickel, zinc, and lead. They will reduce the cost of recovering these metals dramatically from low-grade sulfide deposits largely *in-situ* or heap leaching environments. Those metals and processes most energy intensive and environmentally malign are the most likely to be replaced by these processes, most particularly, the recovery of nickel.

Second, the biological processes that provide for *in-situ* leaching capability, yet undeveloped, will find a solid commercial market. As ore grade declines, it will become increasingly necessary to reduce the energy, labor, and capital costs associated with ore extraction. As recognized by the CEOs in our survey, *in-situ* mining provides the capability of reducing dramatically resource recovery costs. Biological *in-situ* recovery of metals, although slow, requires little capital, energy, or labor costs and is the most environmentally benign of the metal recovery processes. Furthermore, as *in-situ* processes produce less waste rock, the land acquisition and resulting mine development capital costs are reduced. *in-situ* leaching could also become critical if the Environmental Protection Agency declares this gangue rock a hazardous waste. Although the development of new strains of bacteria and processes may enhance the economics of these processes, presently the limiting technology is the lack of low- cost and effective rock fragmentation processes (Ismay *et al.* 1986). More *in-situ* leaching would be cost-effective in North America with presently available organisms if the industry had available these lower cost rock fragmentation methods. It is time now for the mining industry to begin to apply some creative thinking and research monies into new rock fragmentation processes to take full advantage of the many economic benefits of biological *in-situ* leaching. The most widely utilized rock fragmentation technique — blasting — has been utilized for the fragmentation of rock since at least 2000 B.C. (Singer *et al.*, 1968). New technology for such low-cost rock fragmentation may not need to be developed anew, but rather borrowed from other industries and adapted for the resource recovery industry. Both ultrasound and laser technology immediately come to mind, but this should not be considered an exhaustive list.

New or improved organisms for *in-situ* leaching may include genetically-engineered organisms but such organisms and processes are likely to have severe regulatory and scientific hurdles to overcome. The most serious of these is the competition from "wild" native strains of bacteria inhabiting the ore. These will be most acute among *in-situ* leaching environments where the perceived danger of environmental release of an engineered organism and the lack of an aseptic environment accentuate these regulatory and scientific drawbacks, respectively.

Third, considering the growing global awareness of the long-term impact of environmental damage and the nature of the environmental impact of operating and abandoned mining sites, as well as the increasing capability and enforcement by the EPA of private liability for environmental damage, there will be considerable cost advantages in the utilization of "passive" environmental mitigation systems. These are systems that approach or achieve homeostasis where little or no reagents or other inputs are necessary to maintain the system. All necessary energy and/or nutrients are available to the organisms. Some abandoned mine sites we now know are capable of environmental damage for a millennia or more, such as the ancient Roman lead-zinc mines in Wales (Wixson *et al.*, 1985). If both the EPA and private lawsuits are successful in holding mining companies financially liable for these sites, the financial burden to the industry could be untenable. Instead, the industry is likely to seek a technological solution that provides for a mitigation system that can stabilize the abandoned site indefinitely with little or no operating inputs. This will be necessary due to the nature of these sites, as the costs of mitigating a hazardous or toxic effluent in perpetuity with a system with even relatively small operating costs will prove prohibitively expensive. Furthermore, both state and federal regulators are likely to require the establishment of such systems to protect against the insolvency of the company that may leave the state and taxpayers responsible for the long-term protection of the environment.

Fourth, biohydrometallurgy, in its steady state, will encompass several biosorption processes. These processes have the ability to not only remove metals from toxic effluents, but also to recycle the metals and return them to the market and industry. Biosorption processes are capable of removing metals from very large and dilute aqueous solutions, a capability that will become increasingly important as metal concentration standards in drinking water and other waters become stricter in coming years. Although other processes exist for the removal of these metals from solution and recycling, the engineering of biosorption processes hold the greatest promise for reducing costs dramatically. Most importantly, those utilizing the cell envelope and extracellular polymers do not require a living system, which provides the capability to remove metals in varying environments where pH, temperature, metal concentration, etc. can vary and kill a living system. These biosorption polymer systems are likely to employ innovative engineering schemes that provide for durability and simple and inexpensive metal stripping from the polymer.

Furthermore, the application of organisms genetically engineered for growth rate, metal selectivity, and loading factors are likely to play a key role in biosorption, as the issues of regulation and competition with "wild" strains would be mute. The organisms could be grown in an aseptic vat and only the polymers extracted and utilized to sequester metals in an effluent stream, tailing pond, etc. While avoiding the drawbacks of genetically engineered organisms, these biosorption systems could take advantage of the profound benefits. The economics of these biosorption processes will then define the acceptable cost parameters for the primary mining of metals. As the costs of recovering metals from waste streams decline, companies may find it advantageous to "mine" various effluent streams. This will only be advantageous if these recovery costs are less than the market price. Consequently, when the price of a particular metal rises above the cost of recovering it from waste, profit seeking entrepreneurs will enter, "mine" this waste, and return these metals to the market. This increase in supply will then effectively put a ceiling on the price in these metal markets.

Finally, as the environmental mitigation costs of ore processing rise, biosorption processes are likely to be utilized as a primary mineral processing technology. For instance, metal ions in mineral process solutions may be stripped or separated from the process waters by biosorption processes. Polymers, highly selective for one metal and grown from genetically-engineered organisms, would have tremendous utility in the resource recovery industry.

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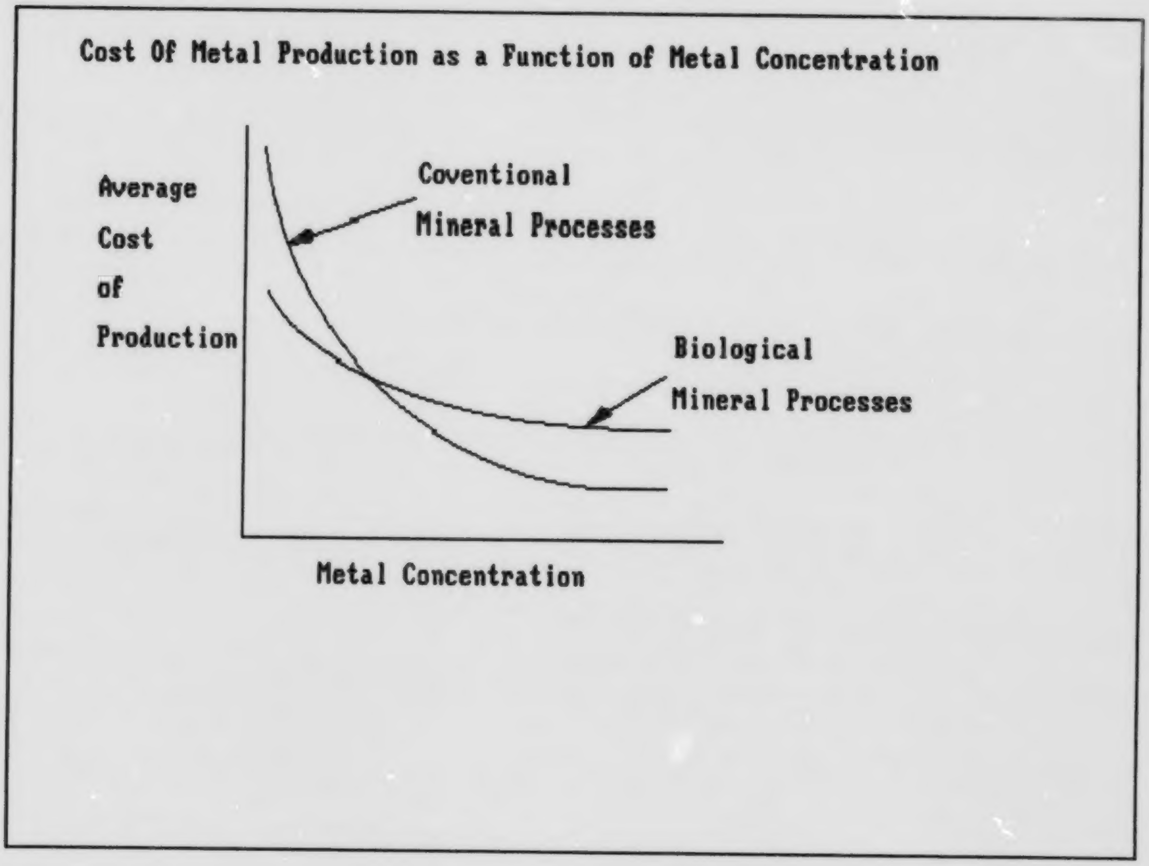

Cost Of Metal Production as a Function of Metal Concentration

Fig. 1

Table 1

Process Economics

	In-situ Capabilities	Lower Energy Costs	Lower Capital Costs	Lower Labor Costs	Convert Waste to Resource	Passive System	New Capabil.	Favorable Environ. Impact	Less Severe Op. Conditions
Copper Leaching	X	X	X	X	X			X	
Gold Ore Oxidation		X	X	X				X	X
Biosorption		X			X		X	X	
Cyanide Destruction		X		X		X		X	X

ENGINEERING DESIGN OF MICROBIOLOGICAL LEACHING REACTORS

L.S. Gormely

Gormely Process Engineering 612 - 1200 West Georgia Street Vancouver, B.C., Canada, V6E 2S9

R.M.R. Branion

Department of Chemical Engineering The University of British Columbia
2216 Main Mall Vancouver, B.C., Canada, V6T 1W5

ABSTRACT

A biooxidation system designer makes use of fundamental knowledge of reaction stoichiometry and kinetics, reactor residence time distributions, mass transfer and heat transfer. This is combined with a laboratory assessment of the biooxidation behaviour of a given feed to result in a full scale design. The general approach is as follows:

1. Review the continuous test program showing the relationship of residence time to degree of oxidation, reagent requirements, and overall metallurgical balances.
2. Define the required extent of reaction and determine the stoichiometry.
3. Calculate process flows for the desired plant scale using pulp densities demonstrated in testwork.
4. From stoichiometry, estimate the oxygen requirement and the heat of reaction.
5. From the process flows and required residence time, size the reactors.
6. From the oxygen requirement, define air flow and dispersion requirements, and select the agitation system.
7. From the heat of reaction, calculate heat balance and size heat exchangers.

This paper reviews the procedures followed in executing such a design.

CONCEPTION TECHNIQUE DES RÉACTEURS DESTINÉS À LA LIXIVIATION MICROBIENNE

L.S. Gormely

Gormely Process Engineering, Vancouver (C.-B.) Canada

et

R.M.R. Branion

Département de Génie Chimique, Université de la Colombie-Britannique, Vancouver (C.-B.) Canada

RÉSUMÉ

Le concepteur d'un système de biooxydation fait appel aux connaissances de base suivantes : aspects stoechiométriques et cinétiques des réactions, temps de séjour à l'intérieur du réacteur, transfert de matière et transfert de chaleur. Toutes ces données sont combinées à l'évaluation des résultats de la biooxydation en laboratoire d'une substance particulière en vue de concevoir un matériel de grandeur nature. L'approche générale est la suivante:

1. Revoir le programme d'essai continu montrant la relation entre le temps de séjour et le degré d'oxydation, les besoins en réactifs et l'équilibre métallurgique global.
2. Définir l'étendue requise de la réaction et en déterminer la stoechiométrie.
3. Calculer le rendement du système compte tenu des exigences commerciales voulues les d'après les densites de pulpe utilisées au cours des essais.
4. À l'aide de la stoechiométrie, évaluer le besoin en oxygène ainsi que la chaleur de réaction.
5. Compte tenu du rendement du système et du temps de séjour nécessaire, déterminer la taille des réacteurs.
6. Compte tenu du besoin en oxygène, déterminer les exigences en matière d'écoulement d'air et de dispersion et choisir le type d'agitateur.
7. Compte tenu de la chaleur de réaction, établir le bilan thermique et déterminer la capacité des échangeurs de chaleur.

Le présent document passe en revue les étapes de la conception d'un réacteur.

ENGINEERING DESIGN OF MICROBIOLOGICAL LEACHING REACTORS

INTRODUCTION

Biotechnology for the mining industry in general, and biooxidation of refractory gold ores in particular, have reached the stage where numerous development programs are being undertaken to evaluate applications for specific orebodies (Bougainville, Riverlea, Tonkin Springs, Sao Bento, Campbell Red Lake, Tundra), and where engineering firms are actively completing technical-economic studies based on these results. The technology is believed to be on the verge of extensive application; it is just waiting for someone to build the first profitable commercial plant.

Figure 1 is a schematic flowsheet of a hypothetical sulphide mineral processing plant incorporating microbiological oxidation of a mineral ore or concentrate. The microbiological oxidation reactor is an integral part of such a process. In it, sulphides are oxidized, permitting dissolution of metal ions and making precious metal residues more amenable to subsequent processing. These oxidation reactions are a result of the activities of microorganisms such as *Thiobacillus ferrooxidans*. While heaps and dumps may formally be considered as such reactors, this paper is confined to consideration of feeds having higher value which require a more controlled process to ensure high metal recovery.

The objectives of the reactor design engineer are to ensure that the system has an adequate residence time to achieve the desired degree of oxidation, while providing a chemical environment conducive to optimal bacterial activity and metallurgical performance. In trying to attain these goals, it is necessary for the designer to select an appropriate reactor system, evaluate the stoichiometry and thermodynamics of the proposed reactions, and estimate the kinetic behaviour of the reactions in the selected system.

In what follows, we present a format for designing a microbial leach reactor which endeavours to take into account the above factors.

DESIGN OVERVIEW

The following points cover the major activities in the selection and sizing of equipment for a biooxidation process:

1. Review the continuous test program showing the relationship of residence time to degree of oxidation, reagent requirements, and overall metallurgical balances.

2. Define the required extent of reaction and determine the stoichiometry.

3. Calculate process flows for the desired plant scale using pulp densities demonstrated in testwork.

4. From stoichiometry, estimate the oxygen requirement and the heat of reaction.

5. From the process flows and required residence time, size the reactors.

6. From the oxygen requirement, define air flow and dispersion requirements, and select the agitation system.

7. From the heat of reaction, calculate heat balance and size heat exchangers.

DESIGN DETAILS

Equipment Size

Determination of the equipment size for a bioleach system requires knowledge of the following:

1. The proposed feed rate.
2. The required degree of oxidation.
3. The mixing characteristics (residence time distribution) of the chosen reactor.
4. The operating pulp density (solids concentration).
5. The kinetic behaviour of the microorganisms with the particular feed selected.

Items 1 and 2 in the above list are based on the economics of the whole mining and metal recovery process. The rate of production is set at the beginning of a study or detailed design; it may be optimised later. The required degree of oxidation is a judgement made by the design engineer where the costs associated with marginal recovery increases are balanced against the increased value. It, too, may be optimised subsequently as part of the on-going design process. Items 3 to 5 are dealt with individually below.

Test Program

The well-known variability of ores and concentrates in terms of chemical composition, crystal structure, and size distribution, coupled with the adaptive characteristics of the mixed cultures used in the process, precludes confident prediction of microbial leach rates from theory or even experience with similar minerals and organisms. Thus, laboratory testing for kinetic evaluation is a prerequisite for a credible design, even at the prefeasibility stage. Our experience shows that these results scale up to commercial operation very well for stirred tanks.

The testing should consist of initial batch amenability runs, followed by operation of a continuous lab-scale reactor system under varying conditions to determine the reactor configuration, effect of recycle, optimum pulp density, pH, residence time, and feed size distribution, the nature of toxic agents in the feed, nutrient requirements, dissolved O_2 limitations and requirement for CO_2. At the same time, other data necessary for design should be accumulated such as liquid-solid separation properties, slurry and solution physical properties, etc.

Batch tests are useful for research purposes, for establishing amenability, and for determining the ultimate extent of oxidation achievable, but we do not believe they can reliably provide the information required to design a plant. During a batch leach, there is continual change in the microbe's environment. In a continuous reactor, steady state in the microbial environment is possible. The bacteria adapt to the steady conditions, and we are able to measure effects resulting from their response to known conditions. Generally, the continuous testing should mimic the proposed industrial design as closely as possible; e.g., if stirred tanks are proposed for the full scale plant, they should be tested in the lab.

Some attempts have been made to model the microbial oxidation system for concentrates by computer (Blancarte-Zurita and Branion, 1988). Inputs are feed size distribution, pulp density, metal content of the concentrate, and an empirical relationship for metal release rate as a function of particle size. Outputs are the extraction rate and the percent extraction as a function of residence time, based on the shrinking particle kinetic model. The program has been used only to predict the results of the continuous leaching of ZnS in the lab-scale reactors described by Sanmugasunderam *et al.* (1986). Figure 2 presents a comparison between zinc extractions as measured in continuous stirred tanks and calculated predictions based on batch shake flask-leach data. Much more testing of this program is required before it can be used in reactor design. To do this, more data is required.

Reactor Mixing Character

A bioleach reactor contains a slurry of mineral concentrate particles which is sparged with air. Agitation is required to keep the concentrate particles in suspension at the pulp densities usually employed, and to provide an adequate level of gas-liquid interfacial area permitting O_2 and CO_2 transfer. To satisfy these demands, the reactor will need to be well agitated, and hence will approximate in behaviour a perfectly mixed reactor, especially in relation to the residence times normally required. For a well mixed three phase reactor, two design concepts are available, the stirred tank reactor, and the air lift (Pachuca) reactor. These are sketched in Figure 3.

Air lift reactors have been used as laboratory scale (up to 10 litre) bioleach reactors by Helle and Onken (1988), Khinvasara and Agate (1988), Acevedo *et al.* (1988), Norris and Barr (1988), and Beyer *et al.*, (1986). A variety of minerals and microorganisms were involved. Pulp densities up to 40% solids were studied. In all cases, some degree of success was observed in microbial leaching. In Acevedo *et al.* (1988), it is shown that for the same air flow rate per unit volume, the O_2 transfer coefficients (k_La values) were somewhat higher in stirred tanks than in air lift reactors. In terms of copper release from chalcopyrite per unit power consumed, stirred tanks were significantly more effective than the air lift reactors. Moresi (1981) has written a paper on the optimal design of air lift fermentors.

Air lift reactors usually are designed with a high aspect ratio to maximize contact with the air that is introduced and keep vertical circulation velocities high. At the tank volumes that are required for typical design situations in biooxidation, the heights become significant. The result is that the air pressure required to overcome the slurry head is high. There is a transition in air supply capital cost at about 15 psig (103.4 kPa) (corresponding to about 30 feet (9.14 m) of water head allowing for some losses) due to the need to switch from blowers to centrifugal compressors.

In our experience, air lift reactors often can maintain adequate solids suspension only when operating in the hindered settling region for pulp density. This region is above the optimum pulp densities encountered for biooxidation.

Our design concept is to provide optimum conditions for oxidation and bacterial growth. Because of the large volume tankage often required, to keep our air pressure below 15 psig (103.4 kPa), we prefer a tank with an aspect ratio of 1:1, which we feel can be most efficiently mixed and aerated with a mechanical agitator.

Pulp Density

Pulp density as used in the mining industry is the weight percent solids in a slurry. In all instances, the economic incentive is to maximize pulp density consistent with satisfactory process performance. The higher the pulp density, the smaller will be the resulting tankage requirement to process a given feed rate for a selected residence time. Further, if metal recovery from leachate is contemplated, higher pulp densities will give higher dissolved metal concentrations, making the recovery more feasible, and the equipment for doing so smaller. (Metal concentrations may also be enhanced by leachate recycle.)

If we assume that the rate of metal release per unit mineral surface area is constant, increasing pulp density or decreasing size should increase the leach rate. However, it is experimentally observed that leach performance deteriorates at pulp densities much above 20 , for reasons that have not been adequately delineated. The same phenomenon has been noted in pressure oxidation. Torma *et al.* (1970) found that bacterial zinc extraction rates increased with pulp density up to 15%, then levelled off, and declined as pulp density rose above 20%. This decline was later found to result from CO_2 limitation. When excess CO_2 was made available, the leach rate began to level off at a pulp density of around 22%. LeRoux and Wakerly (1988) observed a linear increase in the leaching rate of chalcopyrite by *Sulfolbus* in an air lift reactor as pulp density rose to 15%, followed by a linear decrease at higher values. They, too, used CO_2 enriched air.

Pinches (1975) observed an increase in the leach rates of iron from a pyrite-arsenopyrite concentrate with *T. ferrooxidans* as pulp density rose towards 20% in shake flasks. As pulp density increased past 10%, the leach rates rose at a lower rate. While significant leach rates in shake flasks at pulp densities up to 20% have been demonstrated by a wide variety of studies, Liu *et al.* (1988) found that the rate of oxidation of ferrous iron in solution by *T. ferrooxidans* was inhibited at pulp densities between 1 and 5% when glass beads were added to shake flasks. This effect was also observed by Dispirito *et al.* (1981). Liu *et al.* (1988) observed only slight inhibition in a stirred tank at pulp densities up to 15%. Sanmugasunderam (1981) operated a lab scale continuous leach reactor at feed pulp densities ranging from 5.7 to 31.1%; in-reactor pulp densities ranged from 2.5 to 22.4%.

We recommend that the highest pulp density that can be demonstrated in successful continuous test work be used for design. This will usually turn out to be in the range 15 to 20% solids in the feed, with the high end of the range being more acceptable for ores rather than concentrates.

Reactor Configuration

There are limits on the size of reactor in which three phases can confidently be dispersed by current agitation technology. As well, there is some incentive to keep the tank depth below about 30 feet (9.14 m) and its dimensions in an aspect ratio of 1 to 1, if possible, as discussed above. With the slurry feed rates most usually encountered, these constraints result in the system design incorporating more than one reactor.

Another reason for the use of more than one reactor is the desire to achieve high levels of conversion of the sulphide to sulphate. A single stirred tank uses its residence time inefficiently to achieve this goal; a significant proportion of the product is younger than the average residence time, and this results in low conversions (so-called "short-circuiting"). To achieve high conversions, the residence time for a single tank must be much greater than would be needed if the feed could be persuaded to pass through in plug flow, so that it all exited after spending exactly the reactor residence time in the tank. This ideal is more nearly achieved if the residence time is divided up into a number of stirred tanks connected in series.

In the scheme described, if we have (for example) four equal sized tanks in series, the first tank will have a residence time equal to one quarter of the total. We must now examine this time in relation to the the organism's doubling time to see whether we are in danger of washout. If the bacteria wash out of the first reactor, they will wash out of each one in the train sequentially, with consequent process failure. Means of obviating this include increasing the residence time of the first stage relative to the remainder, or recycling bioleachate (which contains bacteria) from a subsequent solid-liquid separation step to the first stage.

To increase the residence time of the first stage, we can increase the tank size, or place two or more equal sized tanks in parallel, which overflow into the rest configured in series. This configuration appears to satisfy all constraints: washout is avoided by providing sufficient residence time in stage 1, and much of the acid produced will be generated where it is most likely to be needed, while the subsequent tanks in series ensure protection against short-circuiting and thus help achieve high conversion.

Stoichiometry of Microbial Oxidation

Table 1 summarizes the stoichiometry of some bioleach systems of interest. Such equations are necessary in calculating the amount of O_2 required per unit of feed, and to estimate the heat released as a result of the oxidation. At a more academic level, they can also be used to estimate the amount of microbial mass generated during leaching, and therefore the amount of nutrients and CO_2 required. For minerals usually encountered, equations are available in the literature (Karavaiko, 1985; and Karavaiko *et al*, 1988); otherwise, they can be inferred from measured changes in solid and solution chemistry in the course of test work.

For example, 10 Mg/d of pure ZnS by equation (5) of Table I requires 6570 kg O_2 per day. This amount must be transferred from air to the leach solution. In practice, excess air needs to be supplied. It is possible to model a reactor and calculate the utilisation, but experience indicates that a value of 20 to 35 percent would apply to a conventional stirred tank reactor. Usually, less than 100 percent oxidation of the sulphide would be attained, and in some circumstances substantially less would be all that is required to achieve the desired results. Thus, the transfer rate for 100% oxidation is adjusted downward to compensate, and the required air supply is estimated based on the adjusted quantity.

Given an appropriate bacterial yield coefficient and knowledge of the microbial elemental composition, the stoichiometry of microbial growth can be calculated. For example, based on the results of Jones and Kelly (1983), we estimate an empirical bacterial formula to be $C_{5.63}H_{10.28}O_{3.06}N$. Sanmugasunderam *et al.* (1986) give a yield coefficient of 5.69 mg bacterial NH_4^+ per g Zn leached. From this we may calculate that 1.16×10^{-1} moles CO_2 and 2.06×10^{-2} moles N must be supplied for each mole of Zn leached. Due to O_2 released in carbon fixation, the O_2 requirement is also reduced from the 2 moles required by equation (5) to 1.88 moles per mole Zn leached. As a matter of design conservatism, it is convenient to ignore this contribution.

There is also a microbial demand for phosphate as a nutrient. We do not have data for the phosphate content of *T. ferrooxidans*, but an average analysis of many microbial species resulted in a value of 1 mole P for every 12 moles N. However, because of unknown contributions from the feed solids and nutrient consuming reactions in the bioleachate, the most common means of determining all the nutrient requirements is to reduce the dosage during continuous test work until process performance is affected.

Heat of Reaction

The amount of heat released in microbial oxidation reactions can be calculated from standard heats of formation for the chemical species in question. Data for solutes is for stated solution concentrations, but corrections to actual anticipated concentrations are not large. Alternatively, heats of formation for the crystalline salts are combined with appropriate heats of solution to obtain the overall heat of reaction. For example, corrected for temperature to 35°C, the value for ZnS oxidation is -202.6 kcal/mole.

Biosynthesis of bacterial cell mass is an endothermic activity, and can be expected to absorb some of this heat. From our empirical formula for biomass, we can write for the stoichiometry of biosynthesis (not a true chemical equation):

$$5.63\ CO_2 + NH_4^+ + 3.14\ H_2O \rightarrow C_{5.63}H_{10.28}O_{3.06}N + 5.68\ O_2$$

In this process, the organism supplies 4 electrons per mole of O_2 released. The heat equivalent per electron is in the range 26 to 31 kcal/electron (Bailey and Ollis, 1986). Taking the upper end of this range, the heat of reaction associated with cell generation is +14.5 kcal/mole ZnS oxidized. This serves to reduce the heat released by about 7%. Again, to be conservative, we ignore the effect when designing the heat removal system.

With feeds having low sulphide content, and thus low "fuel" value, it is conceivable that heating might be required in the first stage of a multi-stage reactor train.

Mass Transfer in Microbial Leach Reactors

The bacteria require dissolved O_2 and CO_2 to complete their metabolic activities. While some CO_2 requirements may be met from carbonates in the feed, some may have to be transferred from air to the leach liquor. Equation (7) quantifies this mass transfer process:

$$N = k_L a(C^* - C) \tag{7}$$

Consider the individual terms in equation (7) with respect to bioleaching. N can be calculated from the stoichiometry as shown above. C^* depends on the temperature, pressure, and composition of the gas and liquid phases. It obeys Henry's law; i.e.,

$$C^* = \frac{P\,y}{H} \tag{8}$$

The Henry's law "constant", H, is actually a function of liquid phase composition and temperature.

If the gas phase is perfectly mixed, as it would approximately be in a well agitated, stirred tank reactor, C^* is the liquid phase O_2 or CO_2 mole fraction in equilibrium with the O_2 or CO_2 mole fraction of the exit gas. If the gas phase is in plug flow as it might approximately be in an airlift reactor, C^* is in equilibrium with a gas having the log mean mole fraction of the inlet and outlet gas compositions.

C^* can be increased, leading to increased transfer rates, by increasing the mole fraction of O_2 or CO_2 in the gas phase. For O_2 transfer, this would involve adding pure O_2, which has not been reported for bioleaching. CO_2 enriched air is frequently used. Torma *et al.* (1972) demonstrated that 1% by volume of CO_2 in air should be sufficiently excessive that dissolved CO_2 would not be rate limiting. Unless excess air is supplied, the mole fractions of O_2 and CO_2 in the exit gas can be seriously depleted due to microbial uptake.

Liu *et al.* (1973) determined that C^* for dissolved O_2 in Silverman and Lundgren's (1959) 9K medium in which *T. ferrooxidans* were growing at 35° under atmospheric pressure was 6.7 mg/dm^3, which is within 5% of the value for pure water. In any event, as long as a consistent Henry's law constant is used for both measuring kLa and using it in design, errors cancel and the predictions will be correct. Thus, it is usually simplest to use values for pure water throughout testing and design.

Bioleach reactors are usually open top, and run at atmospheric pressure. The air is usually introduced near the bottom, so that the head on which C^* is based varies from the full tank depth to atmospheric. Since C^* varies linearly with pressure, we use as an average the value evaluated at one half the slurry depth.

Liu *et al.* (1988) found for *T. ferrooxidans* growing on ferrous iron in solution that if the dissolved O_2 level fell below about 0.2 mg/l, the bacteria would not grow. If it fell below 0.3 mg/dm^3, dissolved O_2 became rate limiting. Myerson (1981) found that dissolved O_2 was limiting in pyrite leaching if it fell below 0.35 mg/l. Pinches *et al.* (1988) observed that dissolved O_2 was not limiting at levels of 0.5 mg/dm^3 and possibly not for values as low as 0.1 mg/dm^3 with mixed culture leaching of pyrite from a gold bearing concentrate. The limiting dissolved O_2 level should be determined in testing for each feed, and design should provide for a level substantially above this. Based on the above data, a level of 1 to 2 mg/dm^3 would be recommended.

We are not aware of any values for the minimum required level of CO_2 in solution for microbial leaching. C^* values for dissolved CO_2 can be found in Ho *et al.* (1987). For design, the most practical approach is to determine the required addition to the air (if any) during continuous testing, and use these results to decide if any facility should be provided in a commercial plant.

Armed with values for N, C*, and C, one can use equation (7) to calculate a value for the kLa to ensure delivery of the required amount of O_2 given the particular driving force (C* - C) selected. Table 2 illustrates the effect of excess air on the mass transfer driving force.

The next step is to choose an agitation and sparger system capable of generating the calculated requirement. Many correlations exist in the literature relating k_La to various operating parameters of the reactor. Most of these are in the form

$$k_La = b \left(\frac{P}{V}\right)^m v_s^n \tag{9}$$

The utility of such correlations is debatable. Charles (1985) has taken four of them and found as much as 40-fold differences in the calculated k_La values for the same power per unit volume and superficial gas velocity. Most published correlations were based on data obtained in small scale reactors where O_2 transfer through the liquid surface at the top of the tank can be a significant part of the total. The geometry of the impellers and the tanks may not scale up proportionately and the correlations are usually based on a Newtonian liquid sparged system. For bioleaching, the reaction mixture has a solid phase as well as gas and liquid phases, and the solution contains high concentrations of electrolytes which tend to inhibit bubble coalescence.

The functional form of equation (9) is probably best used to correlate one's own data for the system of interest.

Mills *et al.* (1987) presented equation (10):

$$k_La = (0.10 - 0.0018\ \emptyset)\ (P_T/V)^{0.67}\ v_s^{0.31} \tag{10}$$

In this dimensional correlation, k_La is in s^{-1}, P_T is in kW, V is in m^3, and v_s is in cm/s. The virtue of this correlation is that the data on which it is based were observed in a three phase system. Its drawbacks are that it was developed using a power measuring technique which is not accurate for small motors and in a small (1.9×10^{-2} m^3) tank. The agitator was a 6 blade flat disk turbine.

Van't Riet (1979) developed equation (11):

$$k_La = 2.0 \times 10^{-3} (P_g/V)^{0.7}\ v_s^{0.2} \tag{11}$$

Units are as before except that P_g is in W, and v_s is in m/s. The advantages of equation (11) are that it is for solutions of electrolytes as would be the case for bioleach reactors, and it is based on data taken in larger tanks (up to 4.4 m^3) with power inputs ranging from 0.5 to 10 kW/m^3. Its disadvantage for application to bioleaching is that it gives no consideration to suspended solids. However, since pulp densities in biooxidation systems tend to be low, this may not be a serious limitation.

Combining correlations of Liu *et al.* (1988a) for k_La and P, with Michel and Miller's (1962) correlation relating power in ungassed and gassed stirred tanks, one can generate equation (12):

$$k_La = 4.92 \times 10^{-3}\ \rho^{-2.8}\ (P_g/V)^{0.93}\ v_s^{0.8} \tag{12}$$

Here, k_La is in s^{-1}, ρ is in kg/dm^3, P_g/V is in W/m^3, and v_s is in m/s. This equation stems from data obtained in a 0.3 m diameter tank in which *T. ferrooxidans* were growing on 9K medium. The suspended solids content was provided by glass beads. The agitator was a 0.1 m diameter 45° pitched 6 blade turbine.

Agitation of Microbial Leach Reactors

Agitation in microbial leach reactors is provided to keep the solids in suspension and to ensure an adequate k_La value for O_2 and CO_2 transfer. Both solids suspension and mass transfer criteria must be considered in the agitator selection. In addition the suggestion has been made by Hardwick *et al.* (1988) and others that high shear agitation might prevent necessary bacterial attachment and/or disrupt the cells, with adverse effects on leaching. This places an upper limit on the level of agitation one can supply to achieve the first two objectives.

Solids suspension is required not only to permit transport of nutrients and O_2 to the mineral surface and products away, but also to ensure a uniform, predictable flow of the solids through the reactor train. The conventional means of achieving level control in a series of stirred tank reactors is to allow them to overflow, one to the other. If a steady state material balance is to be maintained, the overflow must be representative of the tank contents. This implies enough agitation to provide uniform suspension of the solids. Placement of the overflow nozzle is also critical; it must not be located in a quiescent zone; in the lee of a baffle, for example.

Alternatively, one can design for "sand relief"; a means is provided to transfer a proportion of the throughput from near the bottom of the reactor, thereby ensuring that there is no buildup of coarse solids in the reactor. Formation of a fillet in each tank is tolerable, but it cannot continue unchecked without upsetting operations. With the sand relief concept, control over the solids residence time is less secure, and differing residence times may result for differing particle sizes. With gold ores, fillet formation may result in considerable entrapment of free gold, leading to relatively high gold lockup in the circuit.

Mechanical agitators may be classified by the relative levels of shear to pumping that they provide. The typical Rushton 6-blade turbine is classed as a high shear type of impeller, and this is why it is often specified where the desire is to disperse a gas or liquid. Lower on the shear scale, but offering more pumping, are the axial flow turbines, typified by the widely used 45° pitched 4 blade turbine. At the bottom of the scale on shear, but highly rated for pumping are the hydrofoil designs.

We do not believe that a confident selection from among these or other types can be made in laboratory scale studies. At the moment, there is insufficient commercial scale data to make firm recommendations. We recommend that anyone now designing a large scale bioreactor ensure that the design allows for easy changing of impeller types, as this may be an early investigation that is completed in the start up sequence.

It is good design practice to provide internal baffling to prevent swirling and short-circuiting from feed to overflow. Standard anti-swirl baffles are 1/10 the tank diameter in width, set a few centimeters off the tank wall and raised off bottom to prevent formation of dead zones. An inlet baffle leading the tank feed down to agitator level will prevent short-circuiting.

Sparger design needs to be selected in concert with the agitator vendor, and the requirements will be somewhat dependent on the impeller type selected. The sparger is not relied on for bubble break up; it usually is used to release the gas uniformly underneath the impeller at a radius about 80% of the impeller radius. There should be no possibility of off-center sparging, or dangerous radial loads on the agitator could result. We have used both sparge ring and bubble cap concepts. Scale formation has not been a problem.

Heat Transfer

As noted above, significant amounts of heat can be generated in microbial oxidation. If the excess is not removed, the reactor will heat up to the point where the microorganisms are inactivated. In some cases, with feeds of low sulphide content, it may be necessary to heat the feed to the optimum operating temperature. Thus, a reactor heat exchange system is necessary.

The first step is to calculate the anticipated heat load from a reactor by reactor heat balance. The balance should take into consideration:

INPUTS:

1. Heat of reaction
2. Mechanical power input (aeration and agitation)
3. Sensible heat of liquid feed
4. Sensible heat of solid feed
5. Sensible heat of air
6. Heat of dilution of acid

OUTPUTS:

7. Evaporative heat loss
8. Sensible heat of products
9. Heat losses from reactor to surroundings

In an example for zinc sulphide concentrate, we calculated that the net heat load estimated in a detailed analysis agreed within 10% with one which considered only the heat input from reaction and mechanical agitation. This might not be true for the oxidation of a low sulphide feed, when the more careful analysis would be necessary.

Cooling for the reactor could be provided by jacketing, immersed plate-type exchangers, or cooling coils. We have not completed a detailed comparison of these alternatives, but have selected coils because they are widely used, and they offer design flexibility compared with jackets. The major design problem is to find a suitable heat transfer correlation which can be used both with three phase systems and with the agitator design that has been selected.

Shah and Raja Rao (1969) have presented a correlation for heat transfer between an immersed coil and slurries in a stirred tank which was not aerated:

$$(h_oD_T/k_{sl}) = 0.48(D_A{}^2N\ \rho_{sl}/\mu_{sl})^{0.70}(C_p\ \mu_{sl}/k_{sl})^{0.33}\ (H_A/D_T)^{(0.35-0.00049RPM)} \tag{13}$$

where

$$k_{sl} = \frac{k_L\ 2k_L + k_s - 2\ x_v(k_L - k_s)}{2k_L + k_s + 2\ x_v(k_L - k_s)} \tag{14}$$

and

$$\mu_{sl} = \mu_l\ (1 + 2.5x_v + 7.17x_v{}^2 + 16.2x_v{}^3) \tag{15}$$

This correlation was developed for a 4-blade Rushton-type turbine.

Other correlations for stirred tank heat transfer but not specifically referring to slurries or sparged liquids can be found in Holland and Chapman (1966).

For the cooling water flow inside the coil, Kern (1950) recommends use of the Seider Tate correlation:

$$h_lD_c/k_L = 0.027(D_cv\ \rho_L/\mu L)^{0.8}(C_p\mu_L/k_L)^{0.33} \tag{16}$$

In our experience, these correlations estimate too high an overall coefficient for use with axial flow impellers.

Feed Size Distribution

It has often been shown that small particles leach faster than large ones (Torma *et al*, 1972; Sanmugasunderam, 1981; Blancarte-Zurita, 1988; etc.). The principal reason for this is that given the same pulp density, a finely ground substrate has more surface area per unit volume of reactor contents than a coarser one. Grind has a more economically important effect on ultimate recovery because of the need for sulphide liberation (especially with ores), and in some cases the need to stress the crystal lattice to enable bacterial attack (theorized to be the case with chalcopyrite). We recommend that a grind-recovery curve be generated in each instance by lab testwork (batch tests should be adequate), and that in most cases, the feed grind be selected based on an economic comparison of the marginal extraction with the marginal cost of grinding. Process economics are usually less sensitive to the extra capital cost associated with a slower leach rate than they are to the lost value in a leach residue.

Temperature

The optimum temperature for microbiological oxidation of sulphides is usually 35°C, and this should be the design operating temperature for the reactor in the absence of outside constraints. In tropical climates, where cooling water must be recirculated in closed circuit with a cooling tower, this operating temperature may not give a sufficient driving force for heat removal with reasonable heat exchanger area. In such circumstances, it may be permissible to operate up to 40°C, but with little margin for error on the upside.

pH

The optimum pH for *T. ferrooxidans* is around 2 to 3. However, it is believed that in most microbiological oxidation systems, mixed populations are operative. It may be advantageous in some circumstances to operate certain stages of the process at lower pH, and it is believed that this can be done, possibly with some shift in the microbial species mix.

Acid addition to the first stage of leaching may be required to achieve the desired pH range, especially with ores, and this can be a major operating cost item. To minimize the need for this, solid-liquid separation can be conducted at the completion of oxidation, with recycle of the bioleachate to the first stage. Another strategy is to operate with a disproportionately high residence time in the first stage, so that most of the oxidation and acid production is completed there, putting the acid produced in the location where it is required. The subsequent stages act to prevent short circuiting and thereby ensure a high degree of oxidation, where this is required.

Neutralization might be required in the later stages of a process, especially in a concentrate leach, where the feed is capable of little acid consumption, but significant production.

Materials of Construction

In biooxidation systems, we usually encounter ferric iron-containing sulphuric acid solutions in the pH range 1.5 to 2.5. These are very similar to solutions encountered in uranium hydrometallurgy, and generally the same materials selection policies govern. The one difference is that it is understood that a plant in Zimbabwe has encountered problems with toxicity to the bacteria resulting from rubber-lined vessels. This may not extend to all polymeric linings however, and testing should reveal which polymers could make suitable choices. With this caveat, suitable materials could include:

1. Stainless steel. We recommend the use of 316L stainless steel, although it should be possible to get by with 304 as long as the solutions stay oxidizing and chlorides are kept low. By comparison with plastics and polymer linings, stainless steel costs more, but is able to absorb more abuse, and

is generally not harmful to the organisms. The "L" grade of stainless signifies low carbon content and should be specified whereever welding of the material might be required.

2. Fibreglass Reinforced Plastic. These are not all the same, and one should be selected that has been tested for bacterial compatibility and is recommended by the vendor for the proposed service. Fabrication techniques are important, and careful inspection is recommended during the fabrication process. We have not used these in abrasive applications, and so cannot comment on their serviceability in these circumstances.

3. Abrasion resistant polymers and elastomers. As mentioned above, these should be tested for their compatibility with the bacteria, and use probably should be limited to areas where experience indicates that abrasive wear will be excessive.

4. High density polyethylene. This material is generally suitable for piping systems, although it requires support if not used as a liner.

Other choices are available and may be preferred, depending on the economic circumstances and the experience of the designer. Use of test coupons and equipment fabricated from the proposed materials during pilot testing is strongly recommended.

Process Control

Control for this process is relatively simple. The residence times for the process are sufficiently long that most incipient upsets can be spotted and corrected well before they get out of hand. The unfortunate corollary to this is that once a process is off-track, it can take a significant time to come back to specification.

The parameters to control in the reactor system once the process is operating are the pulp density, pH, temperature, and dissolved O_2. Because of the long residence times, continuous measurement is really not necessary, and this greatly simplifies the calibration and maintenance aspects of control. The operator can take these measurements at most two or three times a shift, and have confidence that he has a good picture of the process performance. In a larger plant, it might be desired to adjust the metering of acid automatically according to a continuous pH output, to adjust water flow in the coils according to tank temperature, and to regulate aeration to maintain a desired dissolved O_2 level. The nature of pulp density control would depend on the feed preparation arrangement. There should be no need to adjust the density once the material has entered the oxidation system.

An additional important measurement which is not really controlled, but which provides the best indication of the health of the process is the redox potential or Eh. In these systems, we believe this is primarily a measure of the ferric to ferrous ratio. Empirically, we find it is a good indicator of the extent of oxidation, and the value rises significantly as the ultimate oxidation level is approached. A significant database on Eh correlation with process performance can be compiled during the testing, and this will provide a good check on full scale performance.

CONCLUSION

This paper has outlined the issues to be considered in designing a stirred tank reactor train for biooxidation of a sulphide concentrate or ore. As in most mineral processing applications, there are general principles to be applied, but the use of a natural material as a feed makes the institution of a testing program to develop design data mandatory. We have made frequent reference in our paper on design to the requirement for test data. This should indicate the need to involve a design engineer in the test program at an early stage to ensure that appropriate data is gathered and unnecessary testing or testing on commercially impractical ideas does not take place.

NOMENCLATURE

a = gas-liquid interfacial area per unit volume of slurry

b = empirical constant

C = actual concentration of dissolved O_2 (or CO_2) in the reactor liquid

C^* = concentration of dissolved O_2 (or CO_2) in equilibrium with aeration gas

C_p = specific heat of slurry

C_p = cooling water specific heat

D_A = agitator diameter

D_c = inside diameter of coil

D_T = tank diameter

H = Henry's law "constant" which is a function of liquid phase composition and temperature

H_A = distance from tank bottom to agitator

h_i = inside heat transfer coefficient

h_o = outside heat transfer coefficient for the coil

k_L = mass transfer coefficient

k_L = liquid thermal conductivity

k_s = solids thermal conductivity

k_{sl} = slurry thermal conductivity, calculated by formula in text

N = mass of O_2 (or CO_2) transferred from gas to liquid per unit time per unit volume of slurry

N = agitator speed

P = system pressure

P = power input

P_g = agitator power input to aerated slurry

P_T = agitator power plus bubble expansion power as pressure decreases to top of tank

V = volume

v = fluid velocity in coil

v_s = superficial gas velocity

x_v = volume fraction of solids

y = mole fraction of O_2 (CO_2) in the gas phase

Superscripts:

m = empirical constant

n = empirical constant

Greek:

Ø = volume percent of suspended solids
ρ = slurry density
ρ_L = cooling water density
ρ_{sl} = slurry density
μ_l = liquid viscosity
μ_{sl} = slurry viscosity, calculated by formula in text

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Table 1

Stoichiometry of selected bioleaching processes.

Ferrous iron oxidation in solution

$$4\ FeSO_4 + 2\ H_2SO_4 + O_2 \rightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2O \qquad (1)$$

Pyrite oxidation

$$2\ FeS_2 + 7.5\ O_2 + H_2O \rightarrow Fe_2(SO_4)_3 + H_2SO_4 \qquad (2)$$

Chalcopyrite oxidation (conventional)

$$\begin{aligned}
10\ CuFeS_2 + 42.5\ O_2 + 5\ H_2SO_4 &\rightarrow 10\ CuSO_4 + 5\ Fe_2(SO_4)_3 + 5\ H_2O \\
3\ FeSO_4 + 14\ H_2O &\rightarrow 2\ (H_3O)Fe_3(SO_4)_2(OH)_6 + 5\ H_2SO_4 \\
CuFeS_2 + 2\ Fe_2(SO_4)_3 &\rightarrow CuSO_4 + 5\ H_2SO_4 + 2\ S^o \\
2\ S^o + 3\ O_2 + 2\ H_2O &\rightarrow 2\ H_2SO_4
\end{aligned}$$

$$\begin{aligned}
11\ CuFeS_2 + 45.5\ O_2 + 11\ H_2O &\rightarrow 11\ CuSO_4 + 5\ FeSO_4 + \\
&\quad 2\ (H_3O)Fe_3(SO_4)_2(OH)_6 + 2\ H_2SO_4 \qquad (3)
\end{aligned}$$

BC Research process

$$\begin{aligned}
CuFeS_2 + O_2 + H_2SO_4 &\rightarrow CuSO_4 + FeSO_4 + S^o + 2\ H_2O \\
FeSO_4 + 0.25\ O_2 + 0.5\ H_2SO_4 &\rightarrow 0.5\ Fe_2(SO_4)_3 + 0.5\ H_2O \\
0.5\ Fe_2(SO_4)_3 + 2.33\ H_2O &\rightarrow 0.33\ (H_3O)Fe_3(SO_4)_2(OH)_6 + 0.83\ H_2SO_4
\end{aligned}$$

$$\begin{aligned}
CuFeS_2 + 1.25\ O_2 + 1.67\ H_2SO_4 &\rightarrow CuSO_4 + 2\ S^o + 0.17\ H_2O + \\
&\quad 0.33\ (H_3O)Fe_3(SO_4)_2(OH)_6 \qquad (4)
\end{aligned}$$

Sphalerite oxidation

$$ZnS + 2\ O_2 \rightarrow ZnSO_4 \qquad (5)$$

Arsenopyrite oxidation

$$2\ FeAsS + 2\ H_2O + 7\ O_2 \rightarrow 2\ FeAsO_4 + 2\ H_2SO_4 \qquad (6)$$

Table 2

Driving force for O_2 transfer from air to 9K medium at 35°C and 15% pulp density under 3 m of slurry head, as a function of air utilization.

% oxygen utilization	Oxygen in exit gas (m.f.)	C* (mg/dm^3)	C* - C (C = 1.5 mg/dm^3) (mg/dm^3)
100	0	0	–
80	0.05	2.1	0.6
60	0.10	4.2	2.7
40	0.14	5.8	4.3
20	0.18	7.5	6.0
0	0.21	8.8	7.3

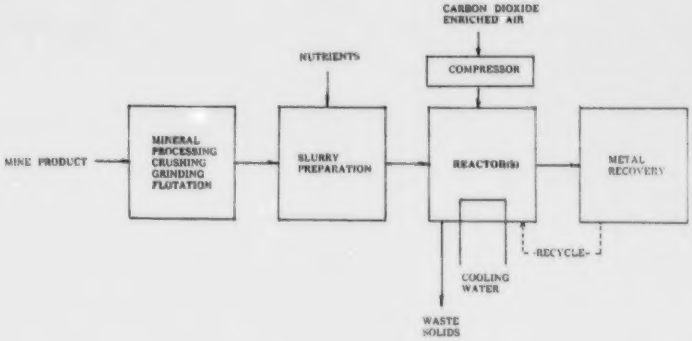
CARBON DIOXIDE
ENRICHED AIR
COMPRESSOR
NUTRIENTS
MINE PRODUCT
MINERAL
PROCESSING
CRUSHING
GRINDING
FLOTATION
SLURRY
PREPARATION
REACTOR(S)
METAL
RECOVERY
RECYCLE
COOLING
WATER
WASTE
SOLIDS

Fig. 1 Schematic flowsheet for hypothetical sulphide mineral processing plant.

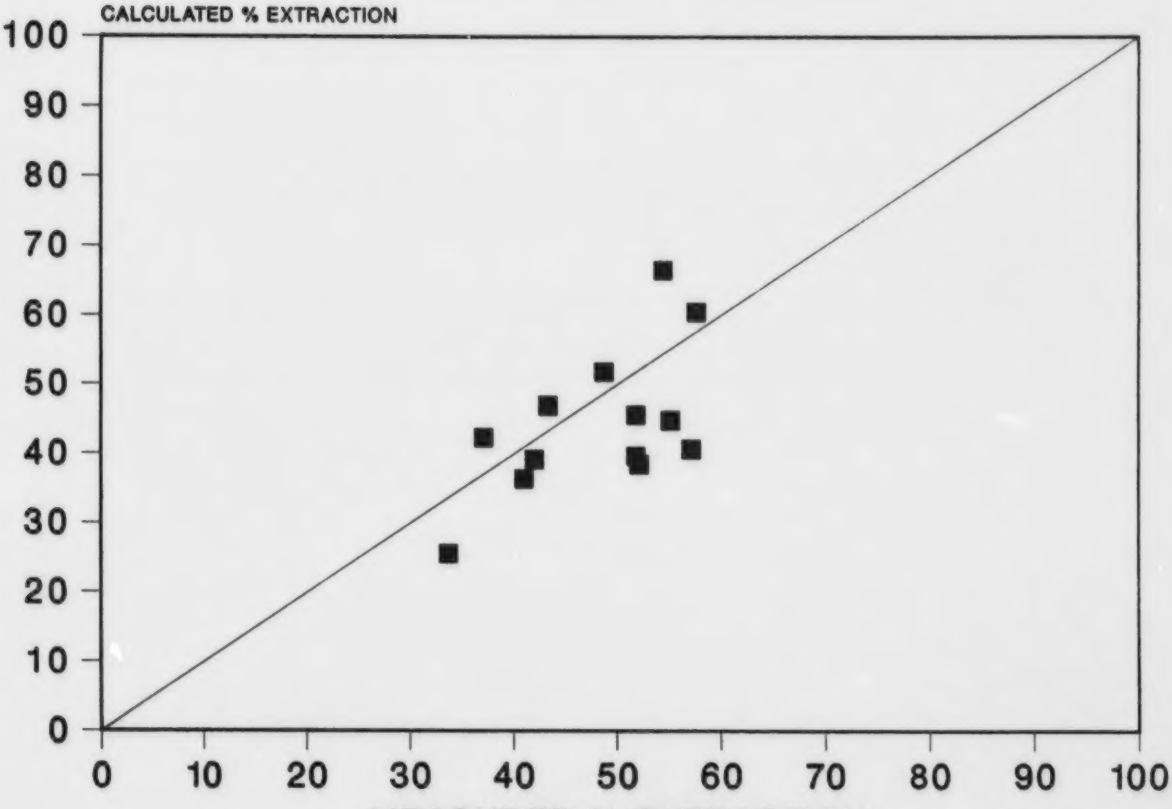

Fig. 2 Comparison between zinc extractions measured in continuous stirred tanks and calculated predictions based on batch shake-flask leach data

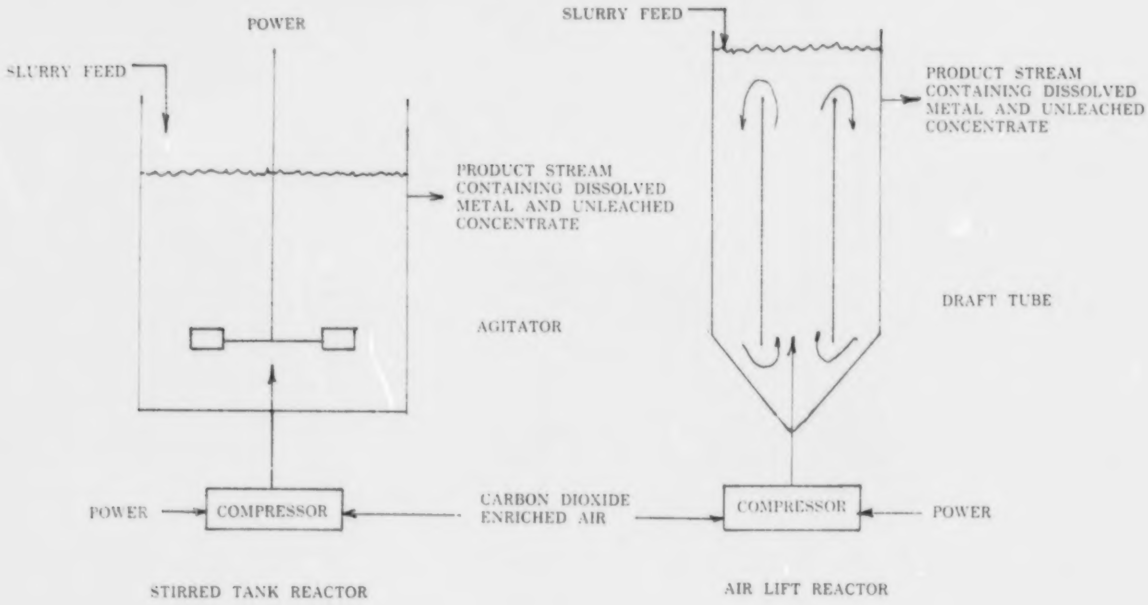
POWER
SLURRY FEED
PRODUCT STREAM CONTAINING DISSOLVED METAL AND UNLEACHED CONCENTRATE
AGITATOR
POWER
COMPRESSOR
CARBON DIOXIDE ENRICHED AIR
STIRRED TANK REACTOR
SLURRY FEED
PRODUCT STREAM CONTAINING DISSOLVED METAL AND UNLEACHED CONCENTRATE
DRAFT TUBE
COMPRESSOR
POWER
AIR LIFT REACTOR

Fig. 3 Sketch of stirred tank reactor and air-lift (Pachuca) reactor.

BACTERIAL LEACHING OF COPPER AND ZINC FROM A SULFIDE ORE BY A MIXED CULTURE OF *THIOBACILLUS FERROOXIDANS* AND *THIOBACILLUS THIOOXIDANS* IN LABORATORY SCALE AND PILOT PLANT SCALE COLUMNS

H.M. Lizama, J.K. Oh, T.L. Takeuchi and I. Suzuki

Department of Microbiology, University of Manitoba Winnipeg, Manitoba, Canada R3T 2N2

ABSTRACT

We have investigated the effects of the particle size of a sulfide ore and the medium flow rate on the leaching rates for Cu and Zn and on the acid consumption in column leaching experiments with a combination of *Thiobacillus ferrooxidans* SM-4 and *Thiobacillus thiooxidans* SM-7.

The metal leaching rates increased with decreasing particle size or increasing specific surface area. Increasing flow rates decreased the acid consumption and increased the metal extraction rates to limiting maximal values.

A large column (55 cm x 4.5 m) leaching set-up was constructed for the scale-up bacterial leaching experiment of 1.64 Mg of 150-200 mm ore. The continuous percolation method did not supply sufficient quantities of air for bacterial leaching of metals or acid production, but the pulsed percolation method led to an efficient leaching of Cu and Zn and eliminated the acid requirement.

LIXIVIATION BACTÉRIENNE DU CUIVRE ET DU ZINC CONTENS DANS UN MINERAI SULFUREUX, SUR COLONNES DE LABORATOIRE ET D'USINE-PILOTE, PAR UNE CULTURE MIXTE DE *THIOBACILLUS FERROOXIDANS* ET DE *THIOBACILLUS THIOOXIDANS*

H.M. Lizama, J.K. Oh, T.L. Takeuchi et I. Suzuki

Département de Microbiologie, Université du Manitoba, Winnipeg, Manitoba, Canada

RÉSUMÉ

Nous avons étudié l'incidence de la grosseur des particules de minerai sulfureux et le débit de la solution sur le taux de lixiviation du Cu et du Zn ainsi que la consommation d'acide au cours d'expériences de lixiviation dans des colonnes par *Thiobacillus ferrooxidans* SM-4 et *Thiobacillus thiooxidans* SM-7.

Les taux de lixiviation du métal ont augmenté sous l'effet d'une diminution de la grosseur des particules ou d'un accroissement de la surface de contact. L'augmentation du débit à provoqué une diminution de la consommation d'acide et une augmentation du taux d'extraction du métal jusqu'à des valeurs limites maximales.

Une grosse colonne de 55 cm sur 4,5 m a été construite pour la tenue d'une expérience de lixiviation bactérienne à grande échelle portant sur 1,8 tonne de minerai de 6 à 8 po. La méthode de percolation en continu n'a pas permis un apport d'air suffisant pour provoquer la lixiviation bactérienne des métaux ou la production d'acide, mais la percolation par coups a donné lieu à une bonne lixiviation du Cu et du Zn et a éliminé la nécessité d'ajouter un acide.

INTRODUCTION

We were investigating the bacterial leaching of Cu and Zn from a sulfide ore containing pyrite, chalcopyrite and sphalerite (Lizama and Suzuki, 1988; 1989) by various *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* strains using shake-flask cultures and small laboratory-scale leaching columns. Based on these studies we selected a combination of *T. ferrooxidans* and *T. thiooxidans* strains isolated from a mine site as the most effective leaching bacteria.

We have now used these bacteria in percolating column leaching experiments of ore samples with different particle sizes. Leaching rates have been found to be proportional to the surface area of ore particles. Increased medium flow rate has resulted in decreased acid consumption.

A pilot plant scale column of 0.6 x 4.5 m has been constructed for the bacterial leaching of 1.64 Mg of 150-200 mm inches ore sample by the percolation method. Details of the experimental set-up, the operation and preliminary results of Cu and Zn leaching are reported.

MATERIALS AND METHODS

Ore samples

All the ore samples were supplied by Hudson Bay Mining and Smelting Co., Ltd., Flin Flon, Manitoba, and contained pyrite, chalcopyrite and sphalerite. The chemical composition of various samples used in this work is given in Table 1.

Bacteria

Thiobacillus ferrooxidans SM-4 and *Thiobacillus thiooxidans* SM-7, isolated from the mine sites, were grown as described previously (Lizma and Suzuki, 1988).

Small column apparatus

A column of 7.5 cm x 59 cm was packed with 1.7 kg of ore with different particle sizes and 1.7 kg of silica sand (20-40 mesh) and percolated with an aerated mixed culture of SM-4 and SM-7 (5% each inoculum) in the HP medium: 0.1 g K_2HPO_4, 0.4 g $(NH_4)_2SO_4$ and 0.4 g $MgSo_4 \cdot 7H_2O$ as described (Lizama and Suzuki, 1989). The flow rate was 0.95 $mm^3/cm^2/s$ in Experiment 1, and was varied from 0.517 to 2.08 $mm^3/cm^2/s$ in Experiment 2. The pH was maintained at pH 2.5 with 10 NH_2SO_4. The total volume of HP medium was 550 cm^3.

Large column apparatus

A pilot-scale percolating column set-up used is shown in Fig. 1. The polyvinyl chloride column (55 cm x 4.5 m) was manufactured by Sceptor Manufacturing Co., Ltd., Edmonton in three 1.5 m sections with flanges. The column was layered with broken granite rock plus glass marbles from the bottom to 200 mm upwards, then was filled with 1.64 Mg of 150-200 mm ore mixed with 650 kg silica sand (20-40 mesh) wetted with water. Layers of geotextiles were placed at strategic locations for uniform distribution of fluid. The bed volume for the ore-sand mixture was 1 m^3. The leaching liquid flowed through the mixture by gravity to the collecting tank, then by a peristaltic pump (Gilson Miniplus 2) to the 50 dm3 culture carboy (Nalgene) where aeration with an aquarium pump and acidification to pH 2.5 with 10 NH_2SO_4 by a Radiometer Titrator TTT11b - pH meter PHM28- magnetic valve set-up took place. A second peristaltic pump carried the liquid to the top of the column where a perforated tubing distributed it evenly over the surface.

Metal extraction analysis

Samples of leaching liquid were filtered, centrifuged and analyzed for metal content by atomic absorption spectrophotometry (Lizama and Suzuki, 1988).

RESULTS

Experiment 1 - Effect of ore particle size

Four ore samples of different particle sizes were used in the small column leaching study as shown in Table 2. The total surface area was calculated from the average diameter and the immersion density. The leaching rate for both Cu and Zn increased with decreasing ore particle size or increasing surface area (Table 2) and the increase was linear with surface area as shown in Fig. 2. The Cu/Zn% extraction ratio increased and the acid consumption decreased with increasing diameter of ore samples (Table 2). During the second 30-day period the extraction rate for Cu and Zn almost doubled and the Cn/Zn% extraction ration increased, but the acid consumption decreased. These results were probably due to the better-established bacterial activities in the columns.

Experiment 2 - Effect of flow rate

Four new small columns were set up with new ore samples of different particle size to study the effect of medium flow rate on the Cu and Zn leaching. The columns were run for two months at the standard flow rate of 0.95 $mm^3/cm^2/s$ in order to establish active bacterial populations before the initiation of experiments.

The results in Table 3 show that the extraction rates for Cu and Zn were lower, but the Cu/Zn% extraction ratios were higher than those in Table 2 due to differences in ore samples. The effects of particle size or surface area of ore were essentially the same. The medium flow rate had a very pronounced effect on the acid consumption, the requirement decreasing with increasing flow rates. It was reduced to near zero at the fastest flow rate used obviously due to an increased acid production by bacteria due to a faster supply of air. The flow rate had an complex effect on the leaching rates of Cu and Zn as shown in Fig. 3. The rates increased with increasing flow rates to maximal values, then decreased. The maximal leaching rates for Cu occurred at flow rates of 1.0 - 1.17 $mm^3/cm^2/s$ in all four columns. The maxima for Zn extraction, however, were achieved at higher flow rates with increasing ore surface areas, i.e., 0.88, 0.88, 1.49 and 1.67 $mm^3/cm^2/s$ for columns 4, 3, 2 and 1 respectively.

Large scale column experiment

A pilot plant scale column shown in Fig. 1 and described in Materials and Methods was percolated with the HP medium. The medium flow rate was 4.5 dm^3/hour or 0.53 $mm^3/cm^2/s$. In addition to water needed to wet the sand (26 dm3), 25 dm^3 of liquid as retained in the column bed and 20 dm^3 of liquid as flowing inside the column at any one time. With the 50 dm^3 of medium in the culture carboy, the total volume of liquid was 121 dm^3. The carboy was inoculated with 4 dm^3 each of *T. ferrooxidans* SM-4 and *T. thiooxidans* SM-7 cultures. As shown in Fig. 4 the pH of column effluent was quite high (5.5), probably due to $CaCO_3$ and $MgCO_3$ in the ore, and the leaching rates of Cu and Zn were low. A further addition of 8 dm^3 of SM-4 and SM-7 culture did not seem to improve the situation.

A number of spills occurred during the experiment, each one followed by a sharp increase in metal extraction. Since the spill led to the drainage of column it was reasoned that the extra air introduced by the spill increased the leaching rates. In order to mimic the effects of spills the column was drained for 24 hours each week for two weeks followed by daily 2-hour stoppage of liquid flow to the column. Fig. 4 and Table 4 show that both procedures increased the metal extraction rates and lowered the effluent pH.

Finally we introduced a "pulsed percolation" procedure by linking the No. 1 pump (culture carboy to column, Fig. 1) to a timer with a four-hour cycle (2 hours on and 2 hours off, Mastercraft Multiple Cycle Timer, Canadian Tire Corp., Ltd., Toronto). It was hoped that 9 dm^3 of medium would flow for 2 hours followed by 9 dm^3 of air increasing the air supply to help the bacterial colonization of the entire column. The volume of total liquid was reduced by 10 dm^3 to 111 dm^3. As is clear from Fig. 4 and Table 4, the metal extraction rates increased significantly. An increased in soluble iron was specially noticeable, probably due to a further lowering of effluent pH. The sulfuric acid consumption up to this point was 0.71 g/kg ore per 30 days. With the pulsed percolation it was reduced to 0.19 g/kg ore per 30 days. After the initial phase of pulsed percolation (days 135-169), 50 dm^3 of the leaching solution were replaced with 50 dm^3 of fresh HP medium in the second phase. The metal extraction rates increased again (Fig. 4, Table 4) and the high rates were maintained after the time shown in Fig. 4 to 990 ppm Cu and 4,250 ppm Zn by day 250, while the effluent pH decreased to pH 2.2, requiring no additional sulfuric acid.

The estimated total surface area of ore available in the large column is derived in Table 5. Since the ore used in the small column leaching experiments was 1.7 kg, the scale ration is approximately 1:1,000 and the surface area of 202,000 cm^2 corresponds to around 200 cm^2 in the small column. Compared to the values in Table 3 the monthly extraction rates achieved with pulse percolation of 0.127% Cu and 0.255% Zn (Table 4) are quite reasonable for the smaller surface area per unit weight or larger particle size of the ore.

CONCLUSIONS

The particle size of ore has a clear effect on the bacterial leaching rate since the total surface area of ore per unit weight increases with the reciprocal of particle diameter, assuming spheres. Miller (1986) reported higher Cu and Zn recoveries at smaller particle sizes in a column leaching test. Laishley *et al.* (1986) found the oxidation rate of sulfur prills by *Thiobacillus albertis* to be a linear function of surface area per unit weight of sulfur. Our results in this paper show that the leaching rates of Cu and Zn from a sulfide ore also increase linearly with increasing surface area in the bacterial percolation column leaching. These results are probably related to the action of bacteria known to attach to sulfide mineral surfaces (Yeh et al. 1987, Bärtels *et al.* 1989).

The most dramatic effect of increasing medium flow rate was the decrease in acid consumption, due obviously to the increased bacterial acid production caused by a sufficient oxygen supply. Increasing flow rates increased the Cu and Zn extraction rates to the maximal values, then seemed to decrease them. In the case of Zn extraction the flow rate, and therefore air supply, required to achieve the maximum increased with increasing surface area as expected.

The large column leaching experiment was successful in supplying essential information in the 1000-fold scale-up from the small column. The shortage of air supply to the column was solved by the pulsed percolation or aeration method, which also eliminated the high pH and acid consumption problem. It was estimated that the external 0_2 supply of 19 dm^3 per 30 days in the continuous percolation could have increased to a maximum of 325 dm^3 0_2 per 30 days in the pulsed method. The final leaching rates achieved for Cu and Zn are quite high considering the large size of ore samples (150-200 mm) used.

ACKNOWLEDGEMENTS

The authors wish to thank the Hudson Bay Mining and Smelting Co., Ltd., the Mines Branch of the Department Energy and Mines of Manitoba and CANMET for a contract under the Canada-Manitoba Mineral Development Agreement. They also thank the Natural Science and Engineering Research Council of Canada for grants to I.S. and a Postgraduate Scholarship to H.M.L., and the University of Manitoba for a Graduate Fellowship to H.M.L.

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TABLE 1

Chemical composition of ore samples

Ore sample	Particle size	Metal content (%)				
		Cu	Zn	Fe	S	Others
Experiment 1						
Column 1	12 mesh - 6 mesh	2.4	9.1	34.7	41.1	12.7
Column 2	6 mesh - 6 mm	4.6	4.1	35.7	39.7	15.9
Column 3	6 - 19 mm	2.3	7.6	30.6	35.5	24.0
Column 4	19 - 25 mm	3.1	15.1	30.2	38.2	13.6
Experiment 2						
Column 1	6 - 13 mm	9.6	12.8	28.0	34.2	15.4
Column 2	13 - 25 mm	9.3	16.2	26.9	33.9	13.7
Column 3	25 - 38 mm	9.1	11.4	27.6	33.4	18.5
Column 4	38 - 50 mm	8.1	12.3	27.5	33.3	18.8
Large column	150 - 200 mm	2.1	3.4	23.0	20.9	50.6

TABLE 2

Effect of ore surface area on the Cu-Zn extraction

	Column 1	Column 2	Column 3	Column 4
Average diameter (mm)	2.8	5.4	14.5	23.7
Total surface area (cm^2)	8083	4200	1627	979
A. First 30 days				
Cu extraction (%/30 d)	1.39	0.93	0.62	0.61
Zn extraction (%/30 d)	5.63	3.31	2.07	1.23
Cu/Zn % extraction ratio	0.25	0.28	0.30	0.50
Acid consumption (g/kg/30 d)	29.2	15.7	14.3	6.5
B. Second 30 days				
Cu extraction (%/30 d)	3.85	2.09	1.49	1.49
Zn extraction (%/30 d)	9.85	7.18	3.33	2.05
Cu/Zn % extraction ratio	0.39	0.29	0.45	0.73
Acid consumption (g/Kg/30 d)	8.5	4.0	4.0	4.0

Flow rate: 0.95 mm^3/cm^2/s.

TABLE 3

Effect of flow rate and surface area

	Columns			
	1	2	3	4
Particle size (mm)	6 - 13	13 - 25	25 - 38	38 - 51
Total surface area (cm^2)	2498	1270	763	557
A. Flow rate at 0.52 mm^3/cm^2/s				
Cu % extraction / 30 d	0.85	0.58	0.33	0.35
Zn % extraction / 30 d	1.04	0.56	0.53	0.45
Cu/Zn % extraction ratio	0.82	1.04	0.62	0.78
g acid/ kg ore / 30 d	3.73	2.95	1.87	1.87
B. Flow rate at 0.95 mm^3/cm^2/s				
Cu % extraction / 30 d	0.97	0.66	0.41	0.45
Zn % extraction / 30 d	1.53	0.64	0.48	0.36
Cu/Zn % extraction ratio	0.40	0.76	0.91	0.90
g acid/kg ore / 30 d	2.03	1.75	1.17	0.75
C. Flow Rate at 1.60 mm^3/cm^2/s				
Cu % extraction /30 d	0.76	0.62	0.43	0.37
Zn % extraction /30 d	1.90	0.82	0.47	0.41
Cu/Zn % extraction ration	0.40	0.76	0.91	0.90
g acid/kg ore /30 d	2.03	1.75	1.17	0.75
D. Flow rate at 2.08 mm^3/cm^2/s				
Cu % extraction / 30 d	0.95	0.55	0.53	0.37
Zn % extraction / 30 d	1.52	0.51	0.42	0.21
Cu/Zn % extraction ratio	0.63	1.08	1.26	1.76
g acid/kg ore / 30 d	0.02	0.02	0.02	0.02

TABLE 4

Leaching rates achieved with the large column

				Pulse percolation	
	Continuous percolation	24 hour drainage	Daily stoppage	Initial	New Medium
g Cu / 30 d	4.3	7.3	11.9	18.6	47.9
g Zn / 30 d	50.3	163.4	50.1	83.3	156.4
Cu % extraction / 30 d	0.011	0.019	0.032	0.049	0.127
Zn % extraction / 30 d	0.082	0.267	0.082	0.136	0.255
Cu/Zn % extraction ratio	0.13	0.07	0.39	0.36	0.50
Initial pH	5.6	5.5	4.4	3.1	2.6
Final pH	5.5	4.2	3.1	2.6	2.6

The column contained 1.65 Mg of 150 - 200 mm ore with 37.8 kg Cu and 61.2 kg Zn.

TABLE 5

Dimensional characteristics of ore in the large column

Total ore used	1,800 kg
Average density	3 g/cm^3
Average particle diameter	17.8 cm
Estimated particle volume (as sphere)	2,953 cm^3
Estimated particle surface area (as sphere)	995 cm^2
Estimated number of particles	203
Estimated total surface area	202,000 cm^2

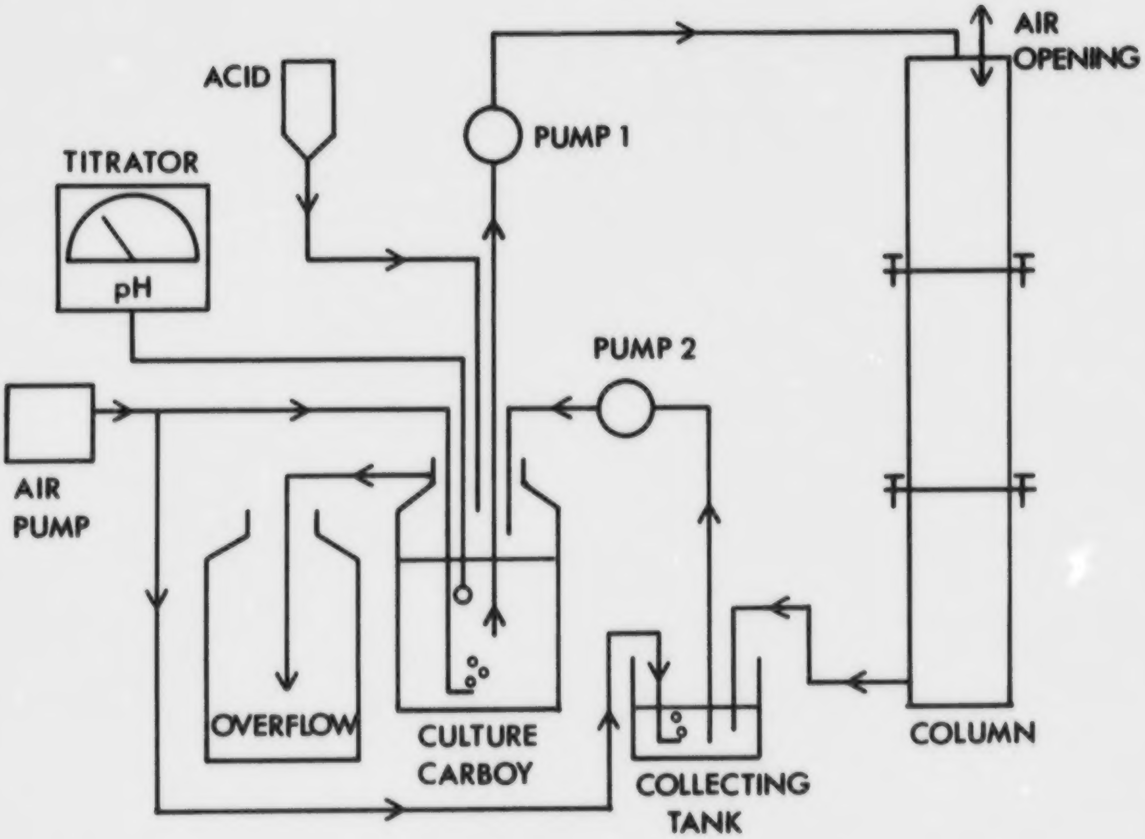
AIR
OPENING
ACID
PUMP 1
TITRATOR
pH
PUMP 2
AIR
PUMP
OVERFLOW
CULTURE
CARBOY
COLLECTING
TANK
COLUMN

Fig. 1 Large-scale column leaching set-up.

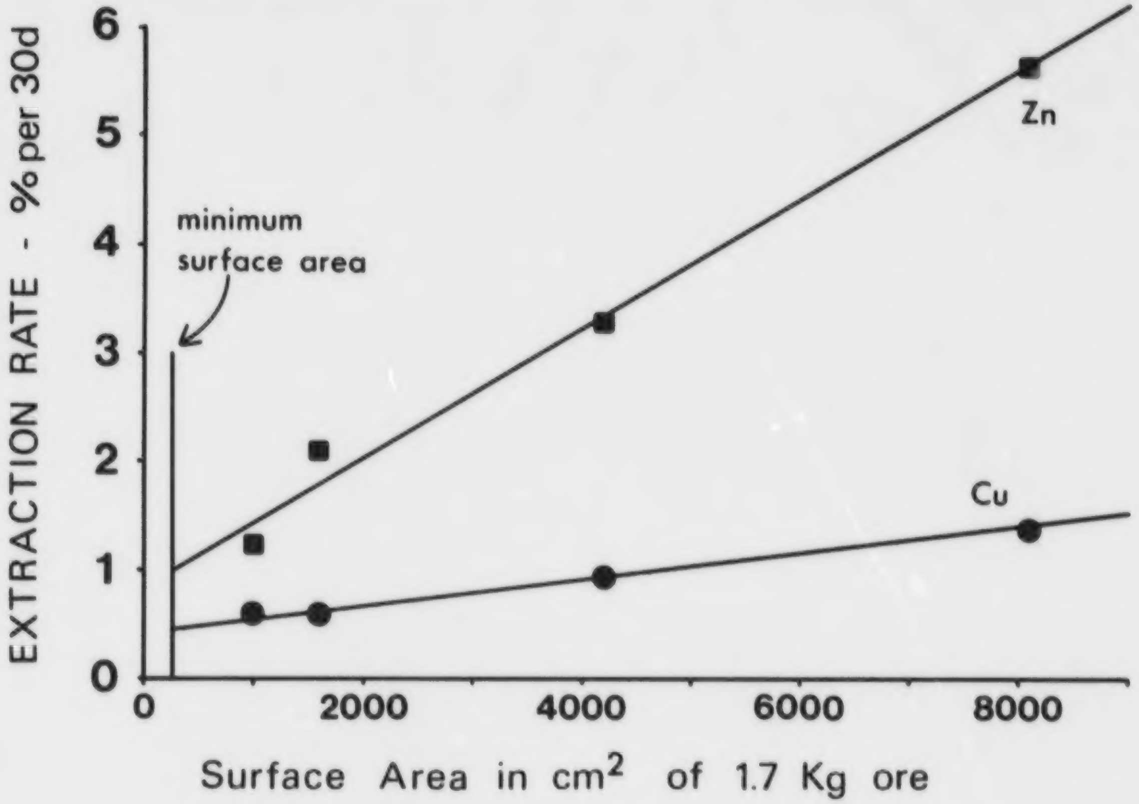

Fig. 2 Effect of ore surface area on the leaching rates for Cu and Zn. The data were obtained from Table 2. The minimum surface area refers to the surface area of a single theoretical sphere of ore.

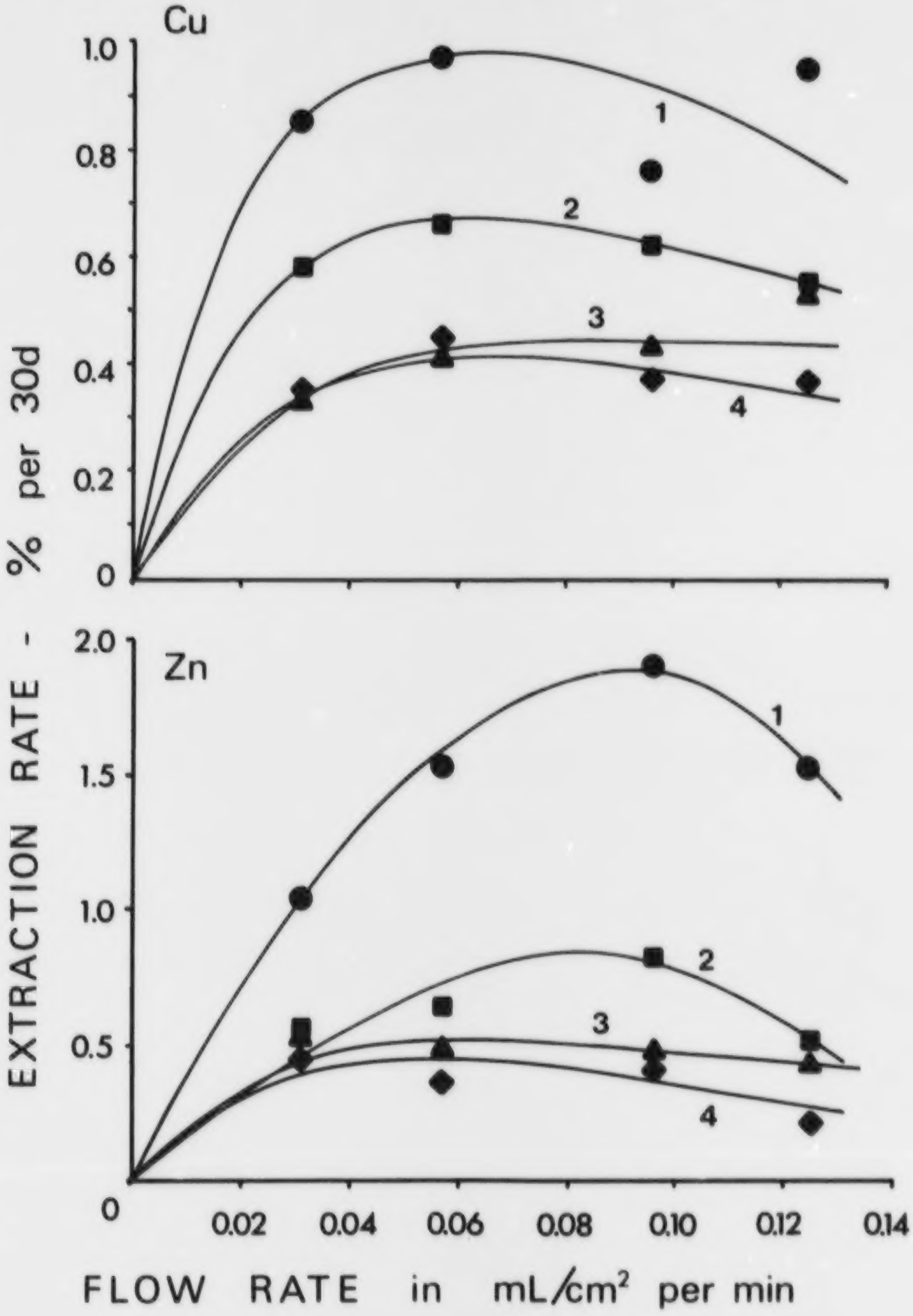

Fig. 3 Effect of flow rate on the leaching rates of Cu and Zn. The data were obtained from Table 3.

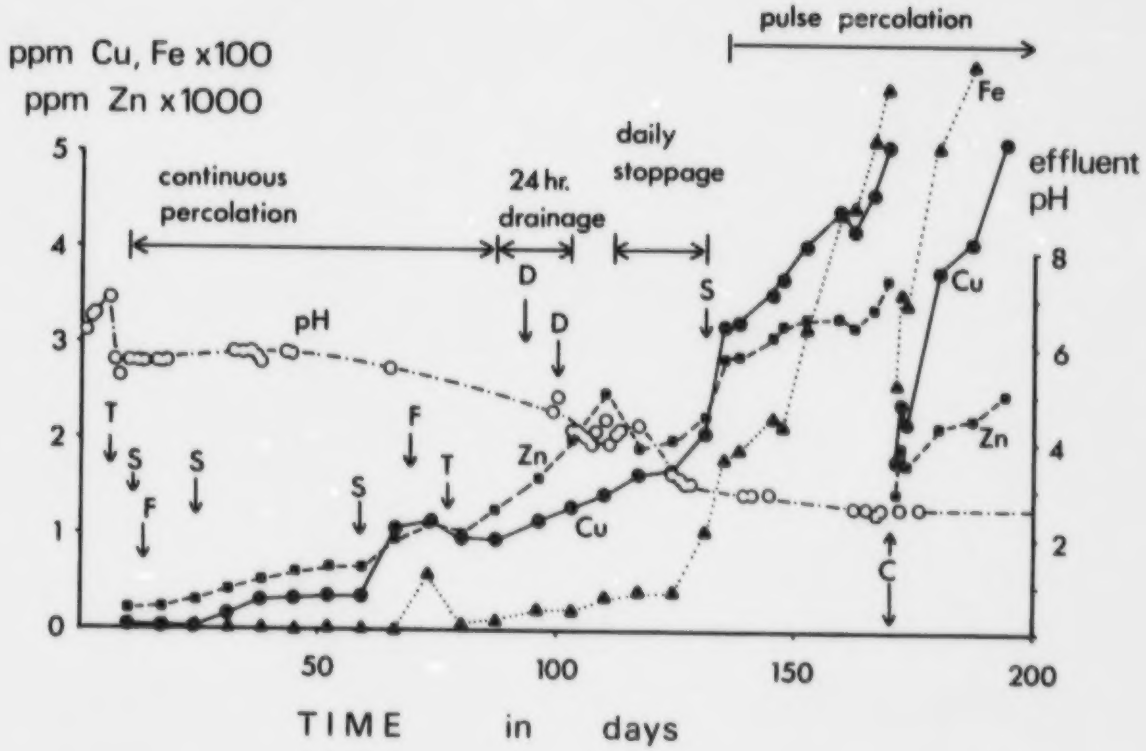

Fig. 4 Time course leaching profile of the large column. T:inoculation with *T. thiooxidans* SM-7. F: inoculation with *T. ferrooxidans* SM-4. S: spill. D: drainage for 24 hours. C: change in medium ($50dm^3$).

BIOLEACHING OF REFRACTORY GOLD ORES – OUT OF THE LAB AND INTO THE PLANT

Ralph P. Hackl and Frank R. Wright

Giant Bay Biotech Inc. 107F, Discovery Park 3700 Gilmore Way
Burnaby, B.C. Canada V5G 4M1

Lynton S. Gormely

Gormely Process Engineering 612 - 1200 West Pender Street
Vancouver, B.C. Canada V6E 2S9

ABSTRACT

Over the past 5 years the authors have been involved in the commercial development of a biological tank leaching process for refractory gold ores. After extensive evaluation on a laboratory scale, the process was operated continuously in several pilot plant campaigns and successfully scaled up during two large scale demonstrations. This paper discusses the various development stages the bioleach process went through and summarizes some of the results obtained during piloting. The operation of a 10 Mg per day demonstration plant at Giant Yellowknife's Salmita Mine is described, as well as the various problems encountered which were unique to bioleaching. In addition, the authors' experience in commissioning a 225 cubic metre capacity, commercial prototype reactor at the Congress property in B.C. is described. This reactor is believed to be the largest bioleach tank tested in the world to date.

BIOLIXIVIATION DES MINERAIS D'OR RÉFRACTAIRES – HORS DU LABORATOIRE ET EN USINE

R. P. Hackl et F. R. Wright

Giant Bay Biotech inc., Burnaby (C.-B.), Canada

L. S. Gormely
Gormely Process Engineering, Vancouver (C.-B.), Canada

RÉSUMÉ

Au cours des 5 dernières années, les auteurs se sont penchés sur la mise au point d'un procédé de biolixiviation en réservoir des minerais d'or réfractaires, en vue d'une utilisation commerciale. Après une évaluation détaillée en laboratoire, le procédé a été mis à l'épreuve en continu dans plusieurs usines-pilotes et il a donné toute satisfaction au cours de deux démonstrations à grande échelle. Le présent document traite des diverses étapes de mise au point du procédé de biolixiviation et il résume les résultats obtenus au cours des essais dans les usines-pilotes. L'exploitation d'une installation d'une capacité de 10 tonnes par jour, à la mine Salmita de Yellowknife est décrite dans le présent document, tout comme les différents problèmes particuliers à la biolixiviation. De plus, l'expérience des auteurs quant à la mise en service d'un prototype de réacteur commercial d'une capacité de 225 mètres cubes, sur la propriété du Congrès, en Colombie-Britannique, est aussi décrite. Ce prototype de réacteur est, à ce jour, le plus gros réservoir de biolixiviation à l'essai dans le monde.

INTRODUCTION

Biooxidation pretreatment of refractory gold/silver bearing ores and concentrates is gaining increasing acceptance as an alternative to roasting and pressure oxidation. Giant Bay Biotech Inc. is one of several firms which are actively engaged in the commercial development of a biological tank leaching process for this purpose. Our research commenced in 1984 with a laboratory program comprised of bacterial strain selection and adaptation, batch bioleach tests, continuous tests and mini-piloting. This was followed by two field trials; the operation of a 10 ton per day demonstration plant at the Salmita mill in 1987 and, recently, the testing of a commercial size bioleach reactor at the Congress property. The purpose of this paper is first to briefly describe the evolution of the laboratory program and relate some of the factors discovered which can impact on bioleach performance. The paper will then go on to discuss the Salmita and Congress projects and highlight some of the scale-up data obtained.

LABORATORY PROGRAM

Culture Development

The laboratory program started with the collection of various strains of bacteria which showed promise for their ability to rapidly oxidize sulphide minerals commonly associated with gold and silver. Cultures were selected for their ability to adapt to a low pH and high concentrations of dissolved metals. Adaptation invariably produced mixed cultures, a trend noted by many others. For example, one of our cultures used for oxidizing mixed pyrite/arsenopyrite concentrates was identified as containing *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum*-like bacteria.

Batch Tests

As a first step in evaluating the applicability of bioleaching for treating refractory ores and concentrates, batch bioleach tests were conducted on various samples obtained from producing and prospective mines. Leaching was typically carried out at a pulp density of 100-300 g/dm^3 in 5 or 10 dm^3 (litre) capacity agitated, aerated plexiglas tanks. This size was found to be convenient in that sample requirements are modest yet enough biooxidized product is produced for several cyanide extraction tests. The solids concentration (pulp density) chosen was dependent on the sample's sulphide sulphur content. An early observation was that bioleaching is sensitive not only to solids concentration (as has been reported by numerous investigators) but also to the slurry sulphide concentration. Consequently, high-sulphide concentrates or massive sulphide ores were generally leached at a lower density than low-sulphide ores.

Batch tests are useful for determining the relationship between cyanide gold recovery and degree of sulphide breakdown. They are simple and inexpensive to perform and results are usually known within 3-4 weeks. If the results are promising, the next phase of testing involves a continuous run.

Continuous Tests

Our first continuous run was initiated in late 1984 on a refractory arsenical concentrate. The apparatus has been modified over the years but basically consists of a feed tank and a series of 5 or 10 dm^3 bioleach tanks. This size is capable of handling 0.5-2 kg/day of feed. Pulp is advanced from one stage to the next by peristaltic pumps. If required, the pH in the first stage of leaching is controlled at 2-2.5 with sulphuric acid. Because of low flow rates, downstream operations such as thickening/filtering, neutralisation and cyanidation are generally performed batch-wise.

Continuous operation provides the optimum conditions for the mixed bacterial population to adjust and adapt to the feed, and often bioleach rates will improve with time. Ideally the feed should be prepared as needed to simulate as closely as possible actual operating conditions. When leaching ore directly, every effort should be made to use only freshly ground ore so air oxidation of the sulphides is minimized. This is particularly important if the ore contains pyrrhotite, which is highly prone to natural oxidation. Our experience has shown that ballmilled pyrrhotite-containing ore stored for even a few days prior to use will oxidize and then appear to bioleach faster than freshly milled ore. This could lead to a gross underestimation of the required retention time. Similarly when leaching concentrates, the concentrate should be fresh so that the possible negative effects of residual flotation reagents on bacterial performance are noticed before the reagents decompose with time.

A properly designed bench-scale continuous run can provide much of the data required for design of a bioleach plant. Process parameters such as solids concentration, number and configuration of leach stages, retention time and reagent consumptions can be determined with a high degree of accuracy. We have found that at least 3 months continuous operation is needed in order to properly define the optimum operating conditions.

Mini-Piloting

In early 1985 a mini-pilot plant was built in order to gain experience in operating on a larger scale. The plant has a capacity of 15-50 kg/day, depending on the feed and the leach tank configuration. The bioleach circuit consists of a 700 dm^3 fibreglass feed tank and four 170 dm^3 plexiglas bioleach tanks. Slurry is pumped from the feed tank to the first stage and advanced from one stage to the next by gravity overflow. Each tank is equipped with temperature controllers which deliver hot or cold water through stainless steel cooling coils situated in the tanks. The tanks are also fitted with control equipment for pH, aeration and CO_2 addition to the air supply.

Biooxidized product slurry exiting the last leach tank is continuously flocculated, thickened or filtered, neutralized, and saved for cyanidation. Bioleachate is neutralised to precipitate the tailings, the tailings are thickened and the neutralized solution is recycled back to the feed tank.

Piloting on this scale was a useful exercise but generated little additional information that could not be provided by the bench-scale apparatus. The plant proved to be too small to provide meaningful scale-up data for agitation, oxygen transfer and heat transfer. One benefit was that agitation shear stress effects, which were not observed in the smaller tanks, did start to become apparent at this scale.

MIXING AND SHEAR STRESS CONSIDERATIONS

In evaluating mixing impellers for bioleach applications one must consider the power, flow and shear characteristics of each. The ideal impeller or impeller combination should be energy efficient, provide sufficient shear for high oxygen mass transfer, and produce good flow to ensure uniform solids suspension. Impellers can be classified into two broad categories, radial flow and axial flow. Radial flow impellers such as the Rushton turbine generate high shear and are therefore good at dispersing oxygen, but they generally have a poor pumping capability and are high power consumers. In contrast, axial flow impellers have a high pumping capacity and consume less power, but generate much less shear.

Both impeller types were evaluated during mini-pilot runs, although it was realized that the information would be of limited use for scale up. The most important finding was that bioleach performance was adversely affected when using Rushton turbines at high rotational speeds, whereas axial flow impellers did not appear to cause any detrimental effect. This strongly suggested that the bacteria could be affected by excessive shear.

Shear stress is an important consideration in many fermentation processes. High shear can rupture the bacteria's cell wall, or, in the case of sulphide oxidizing microbes, possibly hinder the attachment of the bacteria to the sulphide mineral surface. Shear stress is equal to pulp viscosity times fluid shear rate. Shear rates produced by radial flow impellers have been studied by a number of people and the conclusions are somewhat contradictory. Oldshue (1985) states that the impeller zone maximum shear rate for Rushton turbines is proportional to tip speed as given by the formula:

$$S\Gamma\pi ND$$

S = shear rate, s^{-1}
N = impeller rotational speed, s^{-1}
D = impeller diameter, m

If the above formula holds, maximum shear rate would tend to increase with increasing tank size because D generally increases faster than N decreases. Oldshue (1985) recommends keeping tip speed constant when scaling up shear-sensitive processes. However, Bowen (1986) refutes Oldshue and claims that maximum shear rate is proportional to rotational speed and independent of impeller diameter. Bowen suggests that maximum shear rate decreases with tank size because N decreases.

It became obvious that testing of the bioleach process on a large, semi-commercial scale was imperative in order to study the scale-up of agitation and shear stress.

TOXICITY PROBLEMS

Bench-scale and mini-pilot tests provided a wealth of information on toxicity problems. Some of our experience with these are described below.

Flotation Reagents

Most flotation reagents do not appear to affect the bacteria but a few were found to be toxic or inhibitory. Some of the more troublesome ones are:

- cresylic acid
- Aerofloat 31
- Aerofloat 242
- Minerec 2030

Studies undertaken to quantify the toxic levels were complicated by the fact that many flotation reagents decompose with time or are acid-unstable. For this reason batch leach tests are of limited value when testing flotation reagents for toxic effects. Continuous bench scale tests using fresh concentrate are recommended.

If a flotation reagent proves to be toxic, non-toxic alternative reagents should be tried. If this is unsuccessful, regrinding and washing of the concentrate may remove the toxic agent. Acid conditioning of the feed prior to bioleaching can be tried to accelerate reagent breakdown. We have also had some success with bacterial adaptation techniques.

Cyanide Compounds

Certain cyanide compounds are detrimental to bioleaching. This is potentially a very serious problem because in a commercial bioleach/cyanide leach operation it may be necessary to recycle cyanide waste water back to the biooxidation plant. Thiocyanate species in particular are one of the most toxic classes of compounds we have encountered in bioleaching. As little as 5 ppm SCN- can cause a drastic reduction in leach rates. Thiocyanate appears to be stable in acid bioleach solutions, probably because of its ability to form complexes with a wide variety of metal cations. Its presence in a bioleach circuit should be avoided at all costs.

In one test, barren cyanide waste solution containing 294 ppm SCN- was added to the feed tank of a bench-scale continuous run. The dosage was gradually increased and the effect on bioleaching monitored. The results are portrayed in Figure 1. When the thiocyanate level in the feed tank reached

6.3 ppm, the redox potential plummeted and leaching was effectively halted. At this point the contaminated feed was discarded in favour of fresh feed and the bacteria rapidly recovered.

It is not unusual for a typical cyanide tailings stream to contain several hundred ppm SCN^-. The best way to avoid problems with thiocyanate toxicity is to restrict the recycle of cyanide waste water to the neutralization and cyanide circuits and use fresh water and neutralized bioleach solution for the grinding and bioleach circuits. In some cases this may lead to water balance problems. The alternative is to employ a chemical or biological treatment method to reduce the thiocyanate to levels which don't affect the bacteria. Many cyanide gold operations now include a cyanide destruction step, but destruction of relatively non-toxic thiocyanate is usually avoided to save on reagent costs.

We are continuing our research to develop practical methods for detoxifying thiocyanate laden waste solutions. Bacterial adaptation is also being tried but has been met with limited success so far.

Rubber and Hydrocarbons

The preferred materials of construction for large bioleach tanks are stainless steel or rubber-lined carbon steel. We have tested several different common rubber linings and found that some of them appear to inhibit bioleaching. For example, EPDM rubber was detrimental whereas neoprene had no effect. The inhibitory component has not been identified.

Hydrocarbon oils and lubricants can also affect bioleaching, and steps should be taken to prevent their entry into bioleach tanks.

SALMITA PROJECT

In early 1987 Giant Bay and Giant Yellowknife Mines Ltd. embarked on a joint venture bioleach demonstration project at the latter's Salmita mill, located 250 air km northeast of Yellowknife in the Northwest Territories. The objective of the project was to demonstrate technical feasibility on a semi-commercial scale, in a mill environment. Salmita ore was non- refractory and cyanidation routinely gave gold recoveries in excess of 95%. However, Giant Yellowknife's nearby Red 24 claim contained refractory ore. Attempts to process the Red 24 ore through the Salmita mill yielded only 65% gold recovery and resulted in extreme solution fouling. Laboratory tests by Giant Bay indicated that the ore was amenable to bioleaching.

The mine and mill were due to be shut down because ore reserves were exhausted, but before the mill was closed the decision was made to run a 8.9 Mg (metric tonnes)/day (10 stpd) bioleach demonstration plant to treat Red 24 ore.

Laboratory Results

Approximately 500 Mgs of ore were mined and stockpiled for bioleaching. The Red 24 ore is a grey felsic tuff containing arsenopyrite, pyrrhotite and minor amounts of pyrite. The refractory gold is primarily disseminated within arsenopyrite. Pyrrhotite was thought to be responsible for the solution fouling noted during cyanidation. The ore assayed on average as follows:

Gold	21.04 g/Mg
Iron	2.92 %
Arsenic	0.75 %
Total sulphur	0.94 %
Sulphide sulphur	0.87 %

Laboratory testing commenced in December 1986. By May 1987 an intensive program comprising batch tests, two bench-scale continuous runs and one mini-pilot run had been completed. Optimum bioleach process parameters were determined to be:

- 80% minus 200 mesh particle size
- 250-300 g/dm3 (21.5-25% w/w) solids concentration
- three stages of leaching
- 2-2.5 day retention time

These conditions yielded 75% sulphide oxidation and 95% cyanide gold recovery. Gold leached rapidly from biooxidized product and extraction was virtually complete after 8 hours. Cyanide consumption reached 0.9 kg/Mg after 8 hours and 1.5 kg/Mg after 25 hours leaching. Solution fouling did not appear to be a problem.

During the mini-pilot plant run, radial and axial flow impellers were tested in the Stage 1 tanks (hereinafter referred to as tanks 1A and 1B) to compare their effect on bioleach performance. At one point during the continuous run, tank 1A was fitted with a conventional Rushton turbine and tank 1B was fitted with a 45 degree pitched-blade axial flow impeller. Figure 2 shows how bioleach performance was affected by each. The initial tip speed of each impeller was set at 5.3 m/s. Tank 1A, containing the radial flow impeller, experienced an immediate, drastic reduction in bioleach rate as indicated by the Eh (redox potential vs. hydrogen standard). Tank 1B, containing the axial flow impeller, experienced normal leach rates. When tank 1A tip speed was reduced to 3.3 m/s, bioleach rates started to increase and soon returned to normal. The results confirmed our earlier findings that the bacteria are sensitive to the intense agitation produced by radial flow impellers. Not knowing how shear stress would scale up, we decided to try both radial and axial flow impellers in the Salmita plant.

Description of the Salmita Plant

The Salmita mill processed in the range of 150-175 Mg/day of gold ore produced from an underground mine until the start of the demonstration campaign, when the mine was abandoned and allowed to flood.

Briefly, the circuit consisted of crushing, grinding in cyanide solution, thickening, agitation for 36 hours in three wood stave tanks equipped with Dorr agitators, and filtration with washing on two drum filters. Pregnant solution recovered from the thickener was passed through a Merrill-Crowe zinc precipitation circuit for recovery of the precious metal values. Precipitate was shipped to Yellowknife for smelting to bullion at the Giant Yellowknife mill.

For the demonstration campaign, the grinding circuit was converted to grinding in fresh water, with the cyclone overflow being transferred directly to the bioleach circuit. Because of the mismatch in size between the Salmita mill and the bioleach plant, both grinding and cyanidation were done in batches.

A simplified schematic of the biooxidation circuit for the demonstration campaign is shown in Figure 3. The circuit ran for a two-month period from May-July, 1987. Nutrients were added to the feed tank and density was adjusted with water. Feed slurry was continuously recirculated through a head tank, from which branch streams were taken to the first two bioleach tanks. These were operated in parallel and comprised the first of three stages of bioleaching. The first two tanks overflowed to two single tanks in series, making up stages two and three of bioleaching.

Bioleached product overflowed the fourth tank to a three stage neutralisation circuit where acid was neutralised and various ions precipitated through the addition of lime slurry. Neutralised slurry was transferred to the product tank, thence batchwise to the remainder of the Salmita circuit for cyanidation.

Batches of biooxidized product were accumulated in the Salmita thickener to raise the density to a level permitting satisfactory operation of a Dorr agitator. The product was then cyanided in one of the wood stave tanks for 36 hours. The cyanided batch was filtered and washed on the two drum filters, and the solids were discharged to the tailing pond. Filtrate was taken to the pregnant solution tank where it was joined by any thickener overflow (to ensure no gold losses). Pregnant solution was clarified in a vacuum leaf clarifier and the gold recovered by deaeration and zinc precipitation.

Bioleach Tanks and Agitators

Because of time constraints relating to use of a winter road for access, an existing design for a concentrate bioleach plant was used. It was not optimised for low sulphur levels, and thus, the results probably do not represent the best economic performance that could be obtained.

The bioleach tanks were 3.05 m x 3.43 m vertical stirred tanks, arranged two in parallel feeding two in series, for a total of three stages. The tanks were fabricated from 316 stainless steel and were fitted with four baffles and an inlet baffle consisting of a pipe extended close to the tank bottom. The tanks contained stainless heating/cooling coils suspended in the baffles.

Air was admitted to a bubble cap which distributed the air underneath the impeller. All tanks started with agitators fitted with Rushton 6-blade turbines. The shaft, disc, and hub were of stainless steel 316 construction, and the blades were rubber-covered bolt-on type. Axial flow impellers were used later.

Toxicity Problems

Prior to start-up the grinding circuit was thoroughly washed and treated with calcium hypochlorite in an attempt to remove all residual cyanide species. However, it proved impossible to remove thiocyanate totally until a considerable quantity of feed and fresh water had first passed through the circuit. Consequently the bioleach circuit performed poorly at first because of thiocyanate-contaminated feed.

Shear Stress Problems

Bioleach rates in the plant tanks were initially slower than the rates being achieved in comparison lab-scale tank and shake-flask tests. Excessive shear stress was suspected as being the reason. By changing sheaves, different rotational speeds were tried, producing tip speeds varying from 4.0-5.2 m/s, but throughput appeared to be limited to 6-7 Mg/day when using Rushton turbines. This led to the decision to switch first one tank and then all tanks to axial flow impellers. Once this was done, bioleach rates improved substantially. Throughput was increased and steady state operation at a rate of 9.45 Mg/day was achieved without any difficulty. During the last 5 days of operation, throughput was increased to 12-13 Mg/day which was significantly over the design rate of 9.1 Mg/day.

Our findings appear to support Oldshue's contention that radial flow impeller shear stress is related to tip speed, not rotational speed.

Engineering Results

Oxygen Transfer

Once the process problems had been sorted out, a regular program was instituted to measure the mass transfer characteristics of the reactors. The primary focus was the utilisation of oxygen in this system, as this parameter is critical to estimating the quantity of air which must be compressed and sparged into the reactors. Oxygen transfer rates were determined by measuring the oxygen content of the off-gas.

Some of the results obtained are shown in Table 1. At the time the Rushton turbine coefficients were taken, the plant was running at only about 7-8 Mg/day, and it would appear that nearly all the oxygen demand was occurring in the first stage. Thus, the high coefficients expected of a Rushton turbine were obtained in tank 1A. In tanks 2 and 3, the uptake was very low, indicating that little oxidation was taking place, possibly because the bacteria were being affected by excessive shear stress. Under these circumstances, the dissolved oxygen level rises, the driving force is small, and calculation of the absorption coefficient is prone to error.

The observed oxygen uptake rates correlated reasonably well with the oxidation calculated from the sulphur balance. The oxygen demand of the ore was calculated from the head analysis, assuming pyrrhotite and arsenopyrite only. The observed uptake exceeded the computed demand by about 2 percent.

Power Consumption

The agitator power draw was determined by ammeter readings with an assumed power factor. Power draw was also calculated from agitator power numbers supplied by the vendor. A typical draw was about 0.34 kW/m^3 (1.7 hp/1000 US gallons), allowing for a 0.8 power factor. At a daily throughput of 9.5 Mg, the power consumption was 75 kWh per Mg (for agitation only, but including all four tanks). At an average oxygen uptake in stage 1 of 37.5 mg/mm3/s during the balance period, the efficiency of oxygen transfer was about 0.40 kg/kWh (0.65 lb/hp-h), which would be considered low; a value of 2 lb/hp-h is considered achievable in waste treatment (Perry and Chilton, 1973). (This calculation includes only the power for agitation, not that for air compression.) This result is probably due to the constraint of meeting adequate solids suspension, and points to the need to experiment with more efficient pumping impellers in order to achieve uniform suspension with lower power input.

The calculated impeller power draw, based on power factor and rotational speed, was typically half that of the actual draw. It is clear that considerable power was wasted in the motor and gear reducer due to the considerable overdesign resulting from using design parameters for a concentrate to build an ore treatment plant. In a commercial installation, many of these losses would be mitigated by careful design and the greater efficiency of properly sized agitation equipment.

The overall efficiency (tanks 1 to 4) was substantially lower due to the lower uptakes in stages 2 and 3, with no reduction in power consumption. This could be improved in a commercial plant if adequate solids suspension could be achieved with lower power input, since much lower oxygen transfer rates are required in the latter stages.

Heat Transfer

The plant heat transfer design assumed a sulphide concentrate as a feed material, and thus was substantially overdesigned for treating Salmita ore. The flow rates were below the range where reasonable accuracy could be achieved on the instrumentation, and the coil diameters were such that linear velocities on the tube side might provide as much limitation to heat transfer as those in the slurry. This does not mimic industrial practice, and made the data that could be taken of limited value.

Process Results

A summary of the process results achieved during steady state operation at the design capacity is given:

Throughput:	9.45 Mg/day
Retention time:	2.5 days
Solids concentration:	22.5 %
Acid consumption:	8.6 kg/Mg
Lime consumption:	17 kg CaO/Mg
Sulphide oxidation:	75 %
Gold recovery:	95.6 %

Economics

Wright Engineers Ltd. were retained by Giant Bay to complete prefeasibility designs for hypothetical roasting, pressure preoxidation, and biological preoxidation plants located at Salmita and in Nevada, based in part on the results obtained in this campaign. Capital and operating costs were estimated for 1000 ton (892 Mg)/day process plants using each of the three preoxidation methods. A summary of the results (Fall, 1987) is given below:

Salmita Mine, Northwest Territories

	Bioleaching	Pressure Oxidation	Roasting
Capital Cost ($Cdn):	30,800,000	45,000,000	35,000,00
Operating Cost ($Cdn/ton):	42.46	43.53	49.83

Northern Central Nevada

	Bioleaching	Pressure Oxidation	Roasting
Cost ($US):	23,140,000	33,880,000	26,170,000
Operating Cost ($US/ton):	18.67	20.31	19.42

The capital cost is for the process plant building and equipment, and does not include non-process structures, site access, site clearing and preparation, tailings and reclaim system, power, water and sewer supply outside the plant battery limits, and capital cost associated with the mine. The items that have been excluded from the capital cost estimate are site specific and would be the same for all three options.

The operating cost estimate was calculated based on the following items associated with the process plant and equipment: power, fuel, reagents, grinding media, equipment maintenance, and staff, operating and maintenance labour. In the case of Salmita, a daily camp cost per man-day was added.

The Wright Engineers study concluded:

> In both locations, bioleaching had the lowest capital cost and operating cost per ton of ore of the three process alternatives. There is a significant difference in operating cost for all three alternatives when the Salmita location is compared to the Nevada location. The difference is primarily due to the high transportation costs associated with Northern Canada.
>
> Comparing just the three oxidation processes, bioleaching requires fewer pieces of equipment than autoclaving or roasting. Also, the operating conditions required for bioleaching are much less severe than for pressure oxidation or roasting.

CONGRESS PROJECT

Levon Resources Ltd. and Veronex Resources Ltd. in joint venture as the Congress Operating Co. are developing a refractory gold deposit near Goldbridge, B.C. and wished to evaluate the bioleaching technology as it might be applied to their ore. Laboratory tests had shown good potential for improved cyanidation performance if a flotation concentrate were first treated by biological oxidation. To improve confidence in the process and to generate data for an engineering feasibility study, it was determined to operate a full scale commercial prototype leach tank at the mine site.

Because this tank was a full scale prototype, continuous operation would require the purchase and installation of a significant proportion of the equipment needed for the final plant. As agitation studies were the object of the program, it was decided that the required information and confidence could be developed at greatly reduced cost from batch tests.

Description of the Congress Plant

The plant consisted of a series of inoculum growth tanks (the Salmita equipment), the test tank, and supporting aeration, heating and cooling services. The tank was a bolted stainless steel 316L vessel designed to provide close to "standard" geometry usually recommended for agitation studies. The tank was 6.55 m in diameter by 7.31 m high and had a capacity of 225 m^3. This was the size anticipated for a commercial operation. It was outfitted with four anti-swirl baffles located at 90° intervals around the circumference, each 1/10 the tank diameter in width. Air was sparged via a stainless sparge ring situated near the tank bottom. A stainless cooling coil was supported by the baffles.

Because of our prior experience with shear stress problems, radial flow impellers were ruled out for this project. Instead, three different axial flow impeller types were tried.

Feed

The Congress orebody is extremely refractory and the cyanide extractable gold recovery is typically less than 15%. The ore is high in calcite and clay content; sulphides present are mainly pyrite, arsenopyrite and marcasite. Some zones also contain stibnite. Bench scale continuous bioleach tests were performed on both ore and concentrate. Although favourable results were obtained in both cases, bioleaching of raw ore was ruled out because of excessive acid consumption.

Feed to the test tank was a rougher flotation concentrate having the following typical assay:

Gold	17.8 g/Mg
Silver	6.6 g/Mg
Iron	9.45 %
Arsenic	2.24 %
Antimony	1.14 %
Total sulphur	6.31 %
Sulphide sulphur	6.31 %

Slurries had an abnormally high viscosity so tests were run at 150 g/dm3, solids concentration somewhat lower than we would normally run a feed of this sulphide content.

Engineering Results *Oxygen Transfer and Power Consumption*

Oxygen transfer rates were estimated in three ways: by measuring the oxygen content of the off-gas, and by measuring the extent of oxidation accomplished in the pulp as a function of time, both by measuring sulphide destruction and sulphate production. Utilisations were not high, averaging about 15 percent, with the highest observed at about 25 percent.

Power consumptions were determined with a watt-hour meter. Agitation power consumption varied between 30-60 kW (40-80 hp) depending on the impeller type and rotational speed.

A simulation of a reactor was calculated based on the data obtained. At a superficial velocity of 5.0 mm/s (1.1 ft/min), agitation power of 32 kW (42 hp), compression power of 17 kW (23 hp), the assumed absorption coefficient was 32 h^{-1}. The required dissolved oxygen level was assumed to be 2.5 ppm, whence the oxygen transfer efficiency was 0.55 kg/kWh (0.9 lb/hp-h) (energy includes both agitation and compression).

The proposed circuit would require 10 bioleach tanks to treat 113 Mg/day (125 ton/day). If each required the same agitation and air flow as the test tank, the total power for agitation and aeration would be 490 kW (650 hp). The power consumption is then 104 kWh/Mg of concentrate. It may be possible to lower this by using less air and less agitation in the later leaching stages.

Heat Transfer

Heat transfer coefficients ranged from 450-570 $W/m^2/°K$. We used a combination of the Oldshue-Gretton (1954) and Seider-Tate (1936) correlations to estimate a value for the same conditions, and calculated 790 $W/m^2/°K$, assuming a slurry viscosity of 50 cp ($0.5\ N.s.m^{-2}$). It is evident that this correlation overestimates the coefficient, but it was developed for a Rushton turbine, not an axial flow turbine.

Comparison of Lab Scale and Full Scale Biooxidation Rates

Biooxidation rate curves obtained from the test tank were compared to the rate curves obtained with a 5 dm^3 lab scale tank. This was done by taking a 5 dm^3 sample from the test tank immediately after inoculation, and following the oxidation rate for both. The results from one batch test are portrayed in Figure 4. As can be seen, the sulphide oxidation curves were virtually identical, proving that the biooxidation rates achieved in the lab scale tank were duplicated in the full scale tank. Other bioleach indicators such as pH, Eh and dissolved Fe and As, also correlated well.

CONCLUSIONS

A biological tank leaching process for refractory gold ores has been successfully scaled up from the 5 dm^3 lab scale to the 225 m^3 commercial scale, a factor of 45,000 times. Problems unique to bioleaching, such as toxicity effects and agitation shear stress, were encountered and overcome.

Using data obtained from the operation of a 10 ton (8.9 Mg)/day demonstration plant, an engineering prefeasibility study concluded that bioleaching was less expensive than roasting and pressure oxidation. Testing of the process is essentially complete and ready for commercial implementation.

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TABLE 1

SALMITA BIOLEACH PROJECT

SUMMARY OF OXYGEN TRANSFER DATA

Date 1987	Feed Rate mtpd	Tank	Impeller Type	Oxygen Uptake mg/L/h	Sup. Vel. fpm	K_La h^{-1}	% Util.
June 24	7.8	1A	Radial	75	0.10	64	43.2
June 24	7.8	1B	Axial	51	0.16	23	18.9
June 24	7.8	2	Radial	15	0.07	6	12.1
June 24	7.8	3	Radial	10	0.05	9	12.8
June 27	8.0	1B	Axial	105	0.20	40	31.6
June 30	9.3	1A	Axial	113	0.19	41	34.7
July 01	9.8	2	Axial	43	0.09	15	27.2
July 01	9.8	3	Axial	15	0.04	5	20.6
July 02	9.2	1B	Axial	144	0.21	57	40.2
July 02	9.2	3	Axial	31	0.05	12	34.2
July 03	9.6	1A	Axial	143	0.22	57	37.8
July 03	9.6	2	Axial	58	0.09	25	37.5
July 05	9.5	1B	Axial	118	0.21	35	32.5
July 05	9.5	3	Axial	19	0.06	6	17.9
July 06	9.0	1A	Axial	126	0.22	69	34.5
July 06	9.0	3	Axial	26	0.07	10	21.8

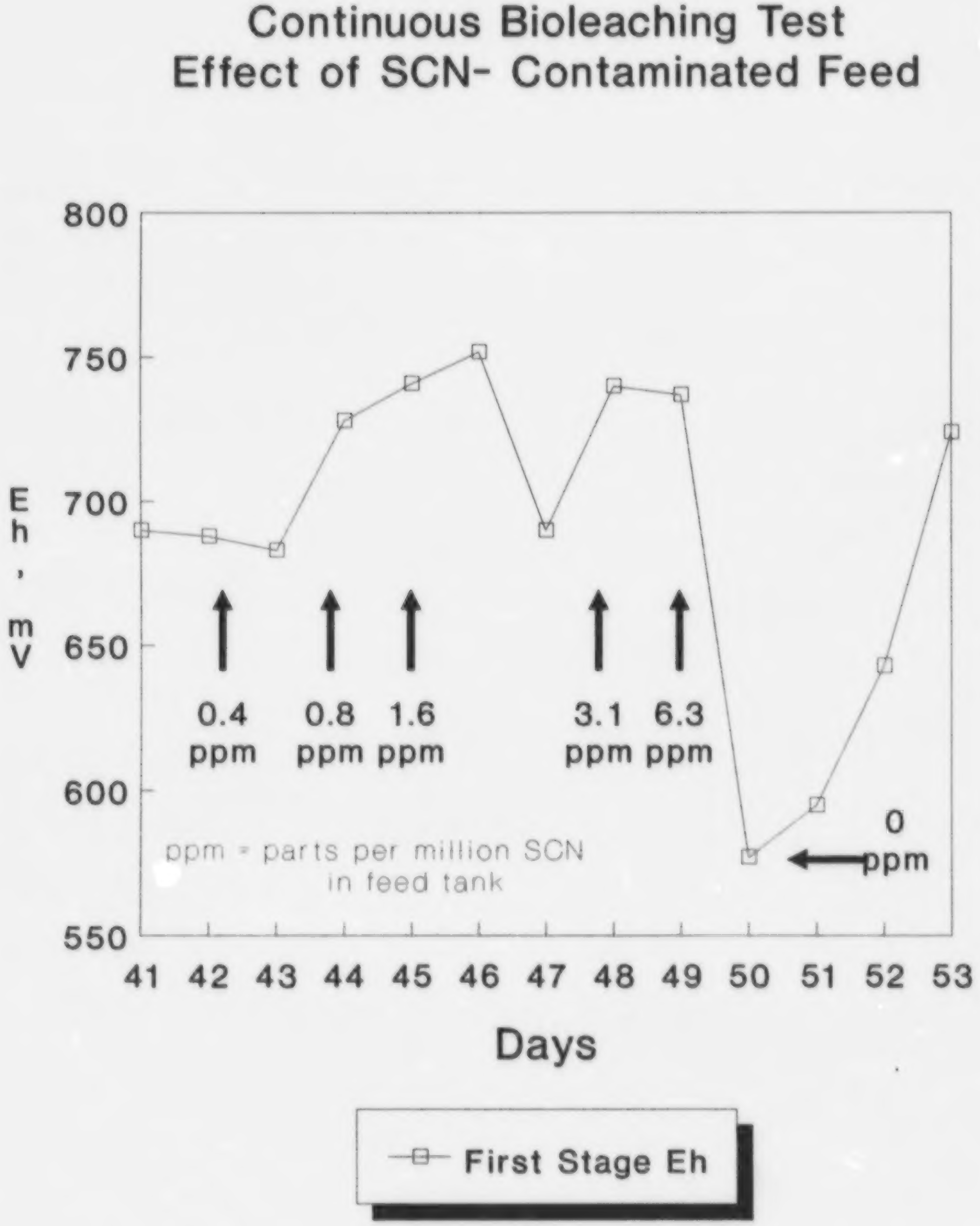

Continuous Bioleaching Test
Effect of SCN- Contaminated Feed
800
750
700
650
600
550
Eh, mV
0.4 ppm
0.8 ppm
1.6 ppm
3.1 ppm
6.3 ppm
0 ppm
ppm = parts per million SCN in feed tank
41
42
43
44
45
46
47
48
49
50
51
52
53
Days
First Stage Eh

Fig. 1

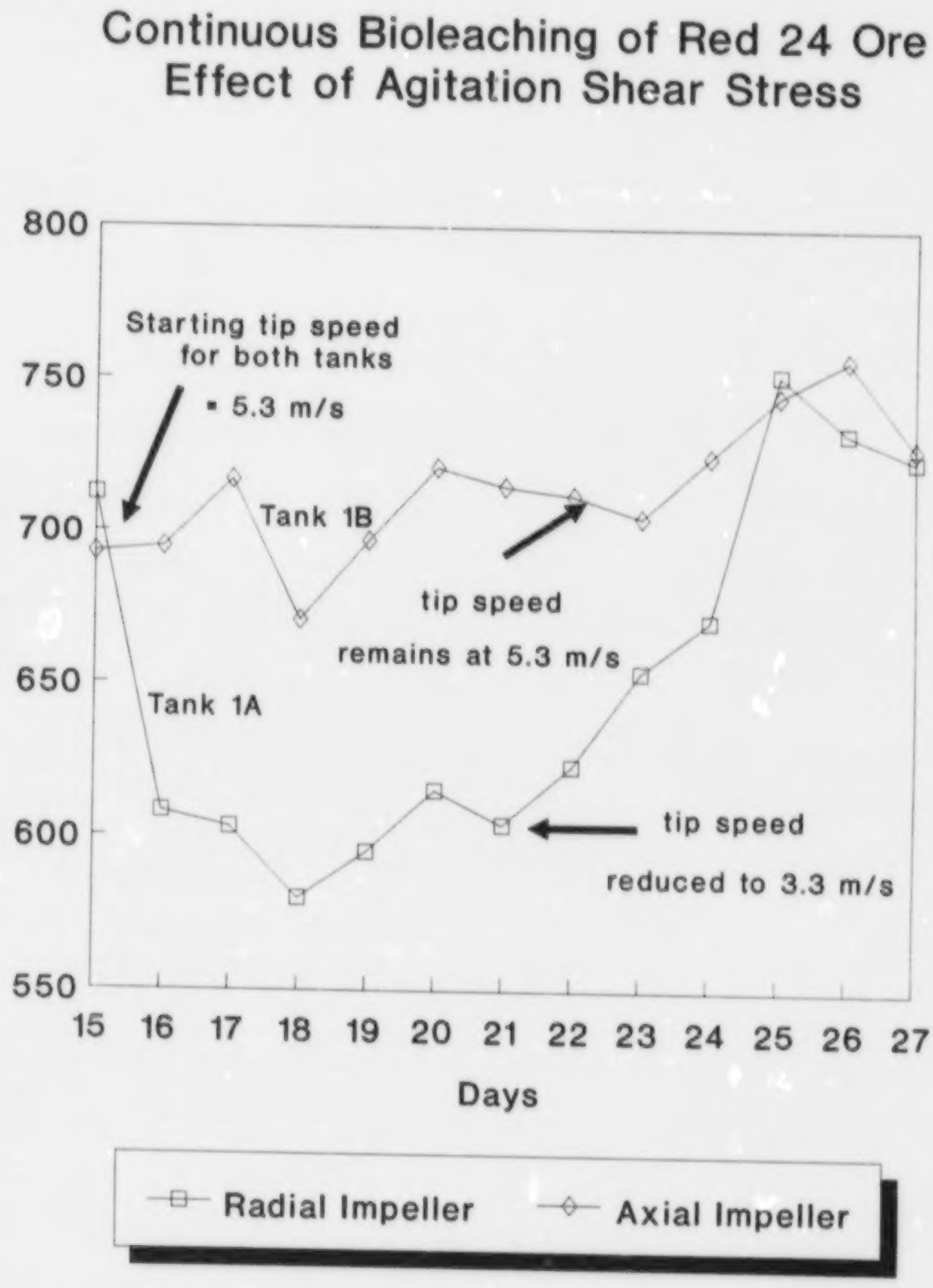

Continuous Bioleaching of Red 24 Ore
Effect of Agitation Shear Stress
800
750
700
650
600
550
Eh, mV
15
16
17
18
19
20
21
22
23
24
25
26
27
Days
Starting tip speed for both tanks = 5.3 m/s
Tank 1B
tip speed remains at 5.3 m/s
Tank 1A
tip speed reduced to 3.3 m/s
Radial Impeller
Axial Impeller

Fig. 2

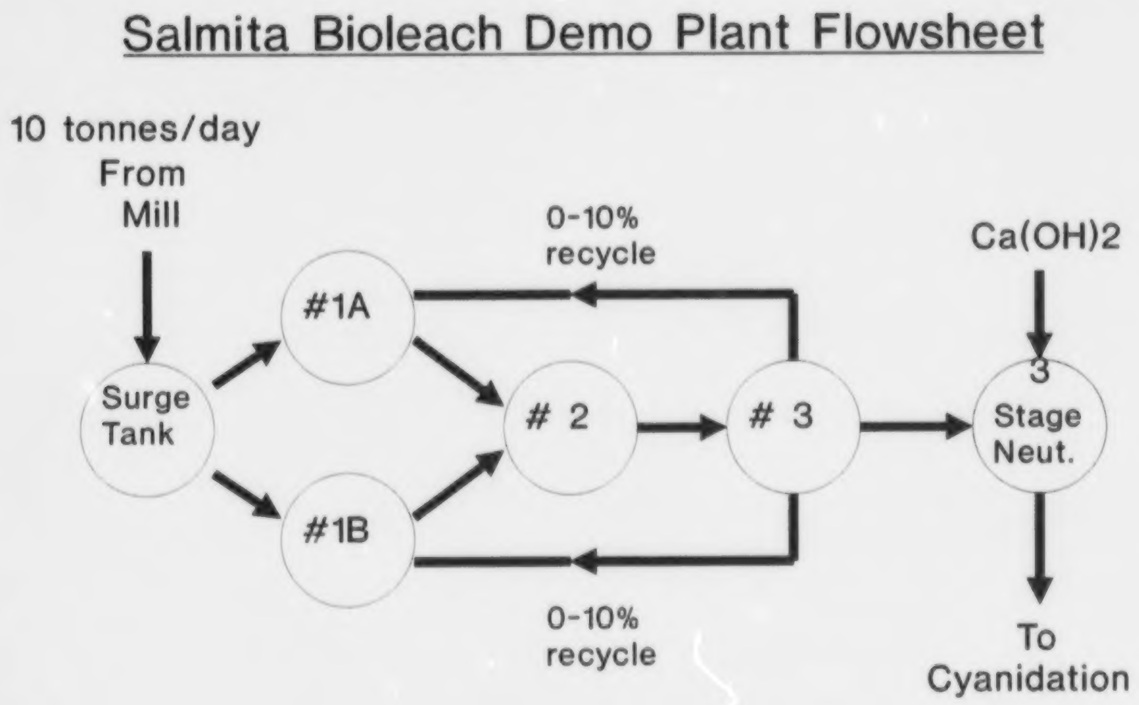
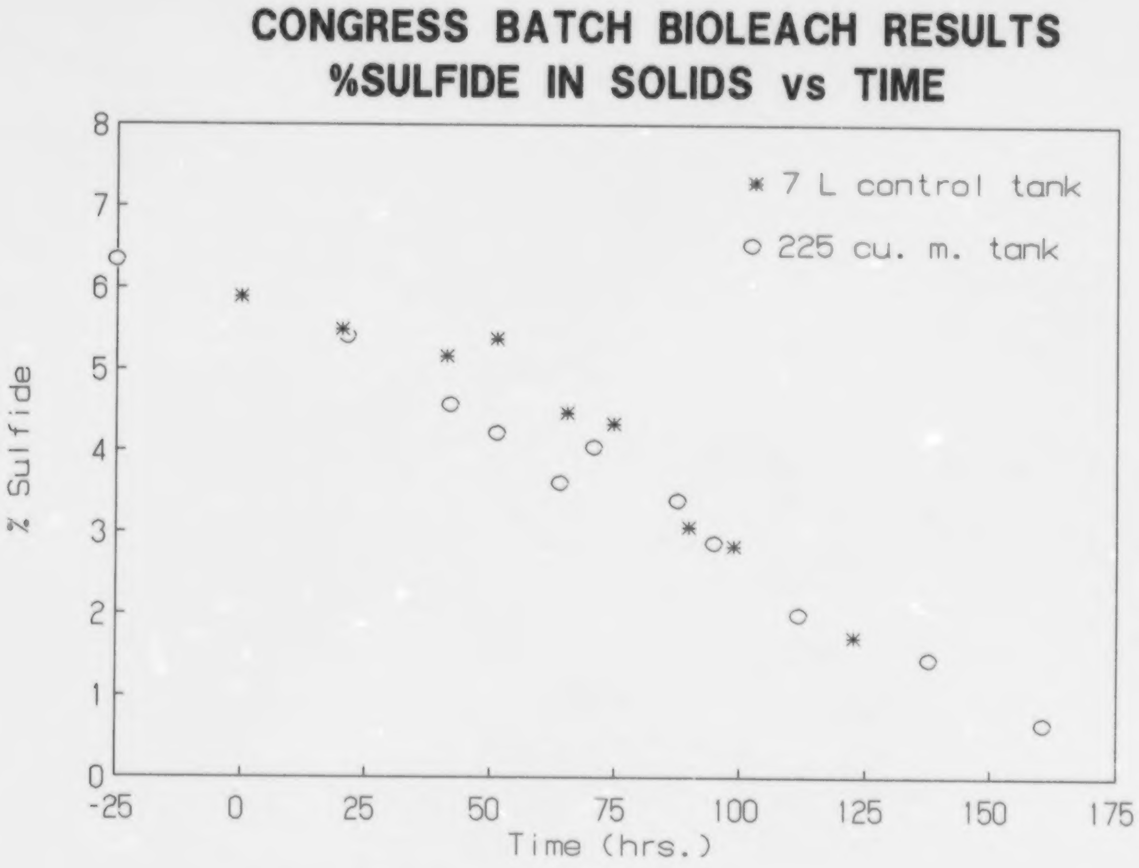

Salmita Bioleach Demo Plant Flowsheet
10 tonnes/day
From
Mill
Surge
Tank
#1A
#1B
# 2
# 3
0-10%
recycle
0-10%
recycle
Ca(OH)2
3
Stage
Neut.
To
Cyanidation

Fig. 3



CONGRESS BATCH BIOLEACH RESULTS
%SULFIDE IN SOLIDS vs TIME

Fig. 4

LIMITATIONS ON PYRITE OXIDATION RATES IN DUMPS SET BY AIR TRANSPORT MECHANISMS

J.W. Bennett, J.R. Harries, G. Pantelis and A.I.M. Ritchie

Environmental Science Program, Australian Nuclear Science and Technology Organisation, Private Mail Bag 1 MENAI, Australia 2234

ABSTRACT

The supply of oxygen to oxidation sites in heaps of pyritic ore is one mechanism which can limit the pyrite oxidation rate. We have modelled gas transport and oxidation in the heap taking account of oxygen transport, heat transport, thermal convection, heap geometry and the removal of oxygen from the heap pore space by oxidation of pyrite in the particles comprising the heap. All the processes depend on physical parameters which can be measured in leach heaps with the possible exception of the process which describes oxidation of pyrite in the particles. We have used values measured by us in the field for most of these parameters.

The model shows that for times less than about two years, diffusion is always an important oxygen supply process. For air permeabilities less than about $10^{-10}m^{-2}$ thermal convection is never a significant air transport mechanism at these early times but it becomes so at permeabilities exceeding $10^{-10}m^{-2}$. At later times, and at permeabilities less than about $10^{-10}m^{-2}$, thermal convection can become significant depending on the magnitude of other parameters such as heap size and pyrite content. For practical sized heaps, bigger than about 0.5 Mt, low heaps oxidise more rapidly than high heaps.

LIMITATION DES TAUX D'OXYDATION DE LA PYRITE DANS DES HALDES ÉTABLIES PAR DES MÉCANISMES DE TRANSPORT AÉRIEN

J.W. Bennett, J.R. Harries, G. Pantelis et A.I.M. Ritchie

Programme de Science Environnementale,
Science Nucléaire et Technologie de l'Organisation de l'Australie,
MENAI, Australia

RÉSUMÉ

L'alimentation en oxygène aux sites d'oxydation des haldes de minerai de pyrite constitue un mécanisme pouvant limiter le taux d'oxydation de la pyrite. Nous avons modélisé le transport du gaz et l'oxydation dans la halde en tenant compte du transport d'oxygène et de la chaleur, de la convection thermique, de la géométrie de la halde et de la suppression de l'oxygène des pores de la halde en raison de l'oxydation de la pyrite contenues dans les particules composant la halde. Tous les procédés sont fonction des paramètres physiques mesurables dans les haldes de lixiviation, à l'exception possible du procédé décrivant l'oxydation de la pyrite contenue dans les particules. Pour la plupart de ces paramètres, nous avons utilisé les valeurs que nous avons mesurées sur place.

Le modèle montre que pour des périodes de moins de deux ans, la diffusion constitue toujours un mode d'oxygénation important. Pour des valeurs de perméabilité à l'air inférieures à environ $10^{-10}m^2$, la convection thermique constitue toujours un mécanisme de transport aérien négligeable pendant ces périodes initiales, mais elle représente un mécanisme très important lorsque les valeurs de perméabilité dépassent $10^{-10}m^2$. Plus tard, lorsque les valeurs de perméabilité redeviennent inférieures à environ $10^{-10}m^2$, la convection thermique peut devenir significative en fonction de l'importance d'autres paramètres, notamment la taille de la halde et la teneur en pyrite. Dans le cas des haldes de taille courante, supérieure à environ 0.5 Mt, celles qui sont basses s'oxydent plus rapidement que les autres qui sont élevées.

INTRODUCTION

An operating sulphidic heap is a highly interactive system. One simple but useful description of the system is to consider it as made up of three interacting processes; transport of oxidant to the particles comprising the heap; transport of oxidant and oxidation of pyritic material within the particles; and transport of the reaction products out of the particles and out of the heap. In this paper we will consider in some detail the interaction between the first two processes but will assume that the interaction of the third with the first two is limited to the effect of water flowing through the heap.

Consideration of the stoichiometry of pyrite oxidation (Brierley 1987) and the comparatively low solubility of oxygen in water leads to the conclusion that oxidant transport to the particles is just the transport of oxygen in air through the pore space of the heap. In most dumps the dominant transport mechanisms will be diffusion due to concentration gradients caused by removal of oxygen from the pore space and convection due to pressure gradients caused primarily by temperature induced density changes in the pore gas. Experimental evidence that convection can occur in real heaps is found in mining wastes at Rum Jungle in the Northern Territory of Australia, see Figure 1.

There have been a number of studies on oxidant transport and oxidation of pyritic material within particles (Box and Prosser 1986; Lin and Sohn 1987; Whittemore 1981) and a number of models flowing from these studies. However for the present study we do not need to consider these models in detail; with little loss of generality we can use for illustration the "shrinking core model" (Davis and Ritchie 1986a, 1986b). This model indicates that the characteristic timescale for oxidising all the pyrite in a particle is $t_p = \mathcal{E}a^2\rho_{sr}/(3\gamma D_2\mathcal{E}_s C_0)$ which is about 0.09 years for a 0.002 *m* particle and typical values for the other parameters. This timescale is typical of leaching rates observed in many reactor and shake flask experiments. The characteristic timescale for convection of gas through a heap with a competing chemical reaction is $t_c = \mathcal{E}\mu_0 L\rho_{sr}/(K\rho^a g\ \beta T_0 c_0)$ which is about 0.43 years, somewhat longer than t_p, while $t_d = \epsilon\rho_{sr}L^2/(c_0 D_a)$ the time scale for diffusion in a heap with a competing chemical reaction is much longer at about 186 years. This means that in many heaps oxygen transport is likely to be rate limiting and that high overall leach rates are likely to occur in heaps where convection is effective. It follows that heap performance may well reflect more the macroscopic physical properties of the heap than the microscopic kinetics of pyrite oxidation.

In this paper we will evaluate heap performance for a range of physical parameters taking proper account of the interaction between the three processes discussed above with a view to providing insight into parameters which are important in determining heap performance.

DESCRIPTION OF INTERACTIVE SYSTEM

In order to approach a more realistic model for the air transport into the heap we include both convection and diffusion so that it c is the concentration of oxygen we have

$$\epsilon_a \frac{\delta c}{\delta t} + \mathcal{E}_a \underline{v}^a \cdot \nabla c - \nabla \cdot (D_a \nabla c) = -S_1(c,\bar{c},T) \qquad (1)$$

where the slid reactant concentration $\bar{c}$ is given by

$$\frac{d\bar{c}}{dt} = -S_2(c,\bar{c},T) \qquad (2)$$

It is well known that the diffusion coefficient D_a is a function of the air porosity $\mathcal{E}_a$. It is adequate to assume that D_a is proportional to $\mathcal{E}_a$ in the range of variation of $\mathcal{E}_a$ appropriate to the present study. The source terms S_1 and S_2 are interrelated and define the macroscopic volume average of the reaction kinetics within each particle. They depend on the local macroscopic transport mechanisms we shall avoid a detailed discussion of the nature of these source terms and simply adopt the shrinking core model as described David and

Ritchie (1986a, 1986b). The temperature dependence is included to describe the biological effects whose activity is influenced by the temperature.

The mass balance for both the air and water phases (f,8,1/pt,1: >a a,w; respectively) is expressed as

$$\frac{\delta \varepsilon_\alpha}{\delta_t} + \nabla \cdot (\rho_\alpha \underline{v}^\alpha) = 0 \tag{3}$$

where ρ_α is related to the intrinsic density ρ^α by $\rho_\alpha = \varepsilon_\alpha \rho^\alpha$. The volume fractions ε_α $\alpha \epsilon \{a, w, s\}$, must satisfy

$$\Sigma_\alpha \varepsilon_\alpha$$

The macroscopic pore velocities for the air and water phases is assumed to be governed by Darcy's Law

$$\underline{v}^\alpha = -\frac{K k_{r\alpha}(\varepsilon_{\alpha_\nabla})}{\varepsilon_\alpha \mu_\alpha} (p^\alpha + \rho^\alpha g z) \tag{5}$$

It is important to note that the relative permeabilities of each studies, e.g. Scheidegger (1957), we simply assume here that the relative permeabilities of each phase is proportional to the porosity of that phase to the third power. This means that the addition of water to the porous matrix, e.g. through irrigation, will reduce the available air pore space (through the constraint (4)), and hence significantly reduce the relative air permeability.

The driving force for the air current within the heap is heat released by the oxidation reaction. Heat released locally may be removed from its site of origin by three major processes; diffusion, convection by air and convection by water. The heat balance equation is given by

$$\Sigma_\alpha \rho_\alpha c_\alpha \frac{\delta T}{\delta_t} + \Sigma_\alpha \rho_\alpha \ \underline{\ }^\alpha \nabla T - \nabla \cdot (D \nabla T) = S_3(c, \bar{c}, T) \tag{6}$$

where S_3 describes the heat of release in the oxidation reactions and is related to S_3 and S_2. We assume that the water phase is incompressible and we relate the intrinsic air density to temperature by

$$\rho^a = \rho^a_0(1 - \beta T) \tag{7}$$

We should keep in mind that (7) is possibly an oversimplification as the air density is also dependent on air pressure and the concentration of oxygen. At high temperatures water vapour may also need consideration.

The intrinsic air density, air pressure, oxygen concentration and temperature are maintained at their respective atmospheric values at the heap/atmosphere boundary. A vertical flux condition is applied at the upper surface of the heap to describe irrigation water infiltrating that surface. The base of the heap is a no flow boundary for oxygen and air but water can drain freely. The heat flux at the base is just the heat removed by water draining at the base.

We constructed a numerical scheme based on a finite difference approximation of the system of equations (1-7) with special consideration given to the changing dominance in space and time of diffusion and convection (we omit details here). As already mentioned we adopted the shrinking core model and have assumed the heap to be comprised of particles of just one size. We see no difficulty incorporating the more realististic particle distribution model of Davis and Ritchie (1987) at a later stage. Although the effectiveness of the microorganisms as catalysts are likely to change as the microbial ecology changes with temperature, for expedience we assumed no dependence on temperature for the catalysed reaction up to a ceiling of 100°C at which temperature the catalysed reaction ceases.

RESULTS

We consider here a cylindrical heap with vertical sides of height $20\,m$ and radius $20\,m$. As a first example the relatively low permeability $K = 10^{-10} m^2$ is used with a particle size a $-0.005\,m$ resulting in the timescales $t_p = 0.56\ y$ and $t_c = 4.3\ y$. Figure 2 shows the evolution of oxygen concentration and temperature in the heap. At year 2 oxygen transport is largely by diffusion from the atmosphere/heap interface which includes the top surface. The inward bending of oxygen contours at the side near the base indicates the onset of convection. At year 3 heat induced airflow has increased and convection in from the sides is clearly evident however air flowing vertically upwards to the top surface works against diffusion directed downwards. By year 4 convection is proceeding strongly but the temperature peak which follows the reacting region moving in from the side has reached the ceiling of 100°C resulting in the local switching off of the oxidation reaction.

Higher permeabilities lead to the earlier establishment of convection and of high temperatures. Figure 3 shows that with $K = 10^{-9} m^2$ convection is well established by one year and that a large region of the heap is at 100°C. The oxidation reaction is switched off in this region which means that oxygen can penetrate through it and fuel oxidation in cooler parts within the heap.

This is typical of many of our simulations at high permeabilities ($K \geq 10^{-9} m^2$). As already pointed out by Cathles and Schlitt (1980) overheating can be curtailed by irrigation. While water percolating through the heap may remove heat, there are two serious considerations which require that the irrigation rate be chosen carefully. High irrigation rates may prevent the induction of convection removing too much heat and by reducing the air permeability through the decrease of air porosity by water displacement in the pores.

Curve (b) in Figure 4 shows the effect on the global oxidation rate (GOR) using an irrigation of $0.01\,m/h$ compared to curve (a) corresponding the same permeability $K = 10^{-9} m^2$ and air porosity $\varepsilon_a = 0.4$ with no irrigation. Some improvement on (b) is achieved at a lower irrigation rate of $0.001\,m/h$ (curve (c)) which allows some convection to occur.

Another important aim of our present studies is to examine the relationship between the oxidation rates at the microscopic level (e.g. oxidation rate of a single particle) and that of the heap as a whole (GOR). In our shrinking core model this can be achieved by varying the particle size. For instance the oxidation rate of a $0.002\,m$ radius particle with 50% of its pyrite oxidised is about 6 times that of a $0.005\,m$ radius particle at the same stage of oxidation. As can be seen from Figure 4 the oxidation rate of a heap made up of the smaller particles is about 45% per year, when the heap is 50% oxidised, compared to about 35% per year for a heap at the same stage of oxidation made up of the larger particles. The modes increase in rate of about 30% indicates that macroscopic transport mechanisms in large heaps tend to dominate microscopic mechanisms.

DISCUSSION

The overall picture seems to suggest that initially oxygen will enter the heap from the atmosphere/heap interface by the process of molecular diffusion and thus confine the reaction to the surface layers of the heap. Only after enough heat has been released from the oxidation reactions will air convection begin to develop. The initial period (or starting up period) preceding the onset of convection is sufficiently long to warrant concern since air convection can be considered to be largely responsible for the acceleration of the GOR when the reaction region moves deeper into the heap. This is also supported by comparing the relative timescale t_c and t_d. The length of this starting up period depends on several factors such as heap geometry, irrigation rate and air permeability.

Heaps in which convection can markedly increase the GOR tend to be relatively high, say greater than 10m, but the aspect ratio may be important. It appears from the results presented that in heaps with air permeabilities less than about $0^{-10}m^2$ convection is not established within the first two years after construction. Detailed heap performance will depend on the value of other parameters such as pyrite density and heap geometry but simulations carried out for a range of these parameters, not presented here, lead to a similar conclusion about the effect of air permeability. For air permeability greater than $10^{-9}m^2$ convection is established rapidly but the GOR may be adversely effected by overheating of the heap. Irrigation will prevent overheating and can improve the GOR but high irrigation may reduce the GOR due to the removal of heat and reduction in permeability as a result of water displacement of air porosity.

Oxygen transport in heaps only a few metres high and tens of metres in radius occurs predominantly by diffusion directed vertically downwards from the top surface. Convection if it occurs is confined to a relatively small region at the sides of the heap. In such heaps the diffusion coefficient, rather than the permeability, is a significant parameter affecting the GOR. Again, high irrigation rates may retard the GOR since the diffusion coefficient decreases with air porosity although not as rapidly as does the air permeability.

It appears that prior knowledge of the physical parameters such as permeability, pyrite content and particle size are essential for evaluating the success of a heap leaching operation. Also a deeper understanding is required of the influence of irrigation rates on the parameters governing the macroscopic transport processes.

We have shown that a 6 fold increase in the oxidation rate at the microscopic level translates to only about 30% increase in the GOR of the heap studied. This suggests that parameters describing the reaction kinetics, as measured say from shake flask or column experiments, are important but cannot be translated readily to describe the oxidation rates of large heaps.

The simulations carried out so far reveal a slightly different picture to that given by Cathles and Schlitt (1980) and others due largely to the inclusion of the process of oxygen diffusion. While the flow paths tend to appear similar (at least after convection takes off) the present model predicts that oxygen penetrates the interior of the heap only as far as a thin reaction region. This penetration occurs along the entire atmosphere/heap boundary which includes the top surface. Highest temperatures tend to be localised around the reaction region moving in from the sides of the heap. Only when temperatures rise so high as to halt the microbially catalysed reaction does oxygen overtake the reacting region and spread further into the unreacted interior of the heap.

NOMENCLATURE

a	particle radius (m)
c	oxygen concentration (kg/m^3)
c_o	oxygen concentration in air (kg/m^3)
$\bar{c}$	intrinsic density of reactant in the solid phase (kg/m^3)
$c\alpha$	specific heat of the α phase ($J\ kg^{-1}\ K^{-1}$)
D	coefficient of heat diffusion ($J\ m^{-1}\ K^{-1}s^{-1}$)
D_a	diffusion coefficient of oxygen in air in the heap (m^2/s)
D_2	oxygen diffusion coefficient in water (m^2/s)
g	acceleration du to gravity (m/s^2)
$K_{r\alpha}$	relative permeability of the α phase

K	permeability (m^2)
L	characteristic length scale of heap (m)
p^{α}	pressure of the α phase ($kg\ m^{-1}s^{-2}$)
T	temperature relative to atmosphere (K)
T_o	characteristic scale for the variable T(K)
t	time(s)
$\underline{v}^{\alpha}$	macroscopic velocity of the α phase (m/s)
z	height (m)
β	coefficient of thermal expansion (K^{-1})
γ	a proportionality constant encompassing both Henry's law and gas law
ε	mass of oxygen used per mass of solid reactant in oxidation reaction
ε_{α}	volume fraction of the α phase
ρ_{α}	density of the α phase (kg/m^3)
ρ_{α}	intrinsic density of the α phase (kg/m^3)
ρ_a^o	*density of air* (Kg/m^3)
$\mu_{\alpha\alpha}$	viscosity of the α phase ($kg\ m^{-1}s^{-1}$)

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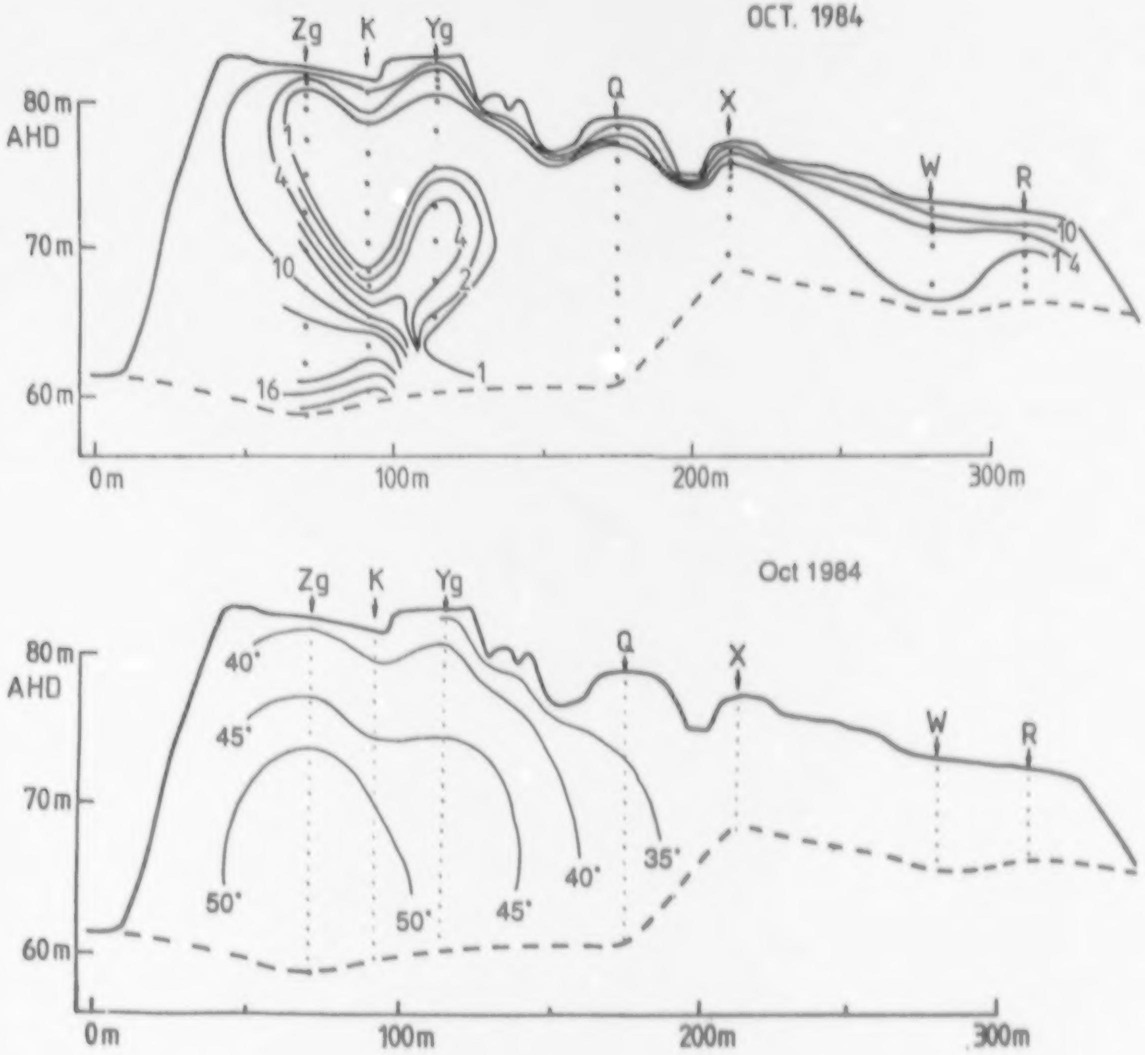

Fig. 1 Measured oxygen concentrations and temperatures in the Intermediate mine overburden heap at Rum Jungle.

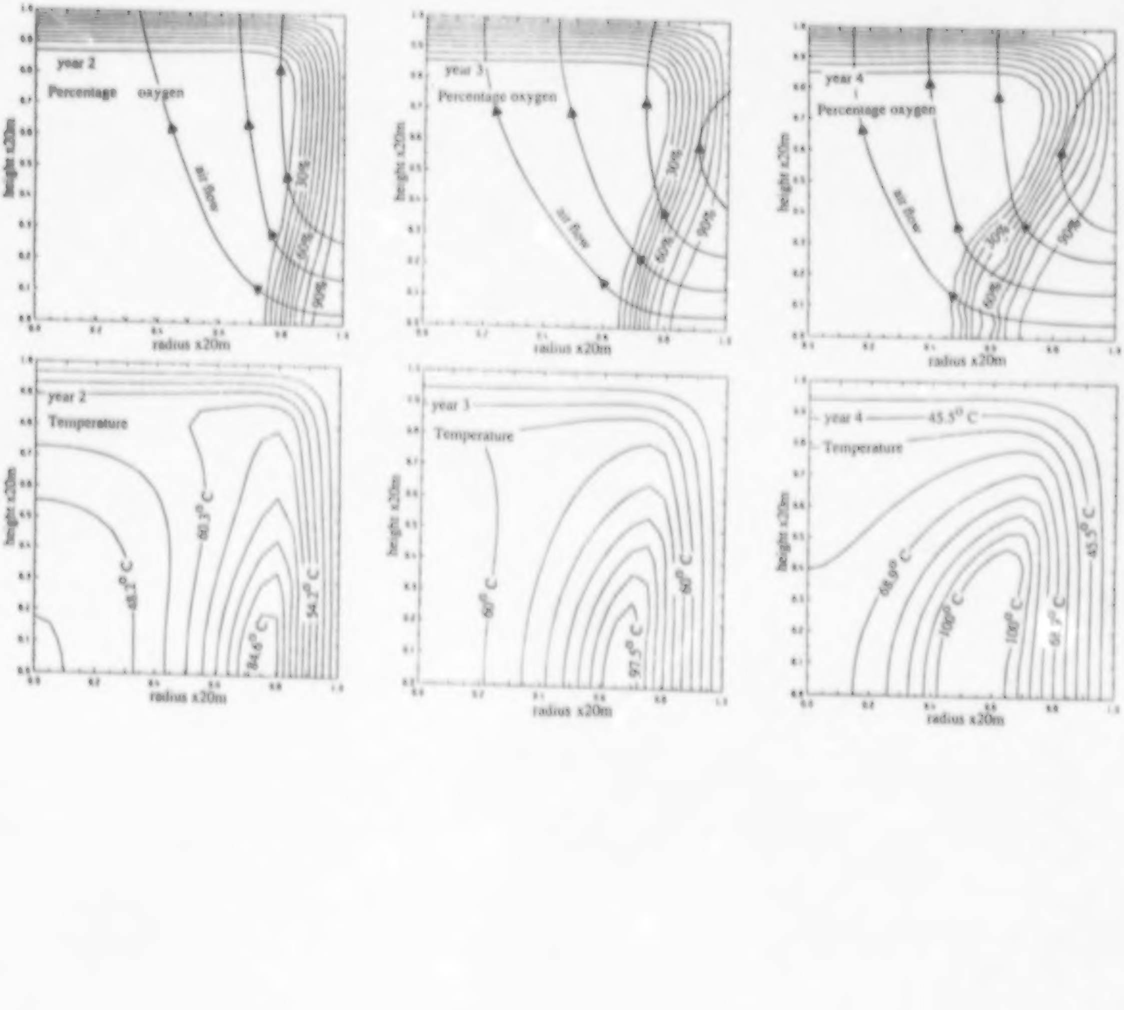

Fig. 2 Oxygen content and temperature contours in a cylindrical heap at years 2, 3 and 4. The permeability $K = 10^{-10} m^2$ and the particle size $a = 0.005\ m$. Arrowed solid lines indicate air flowpaths.

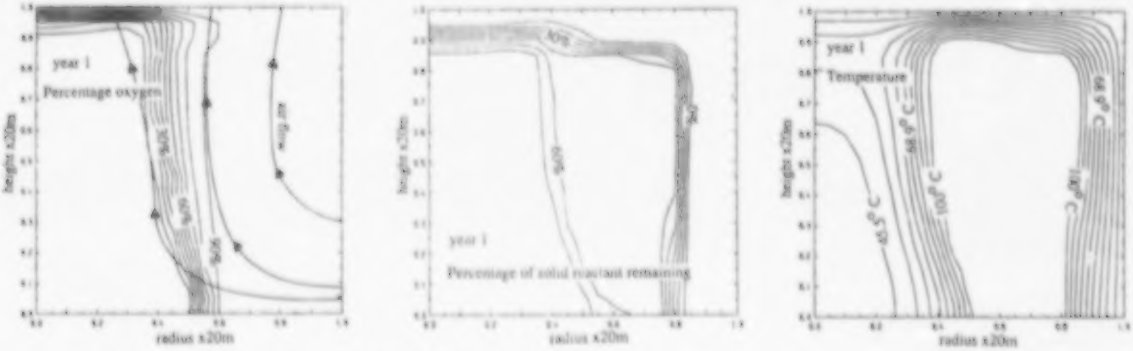

Fig. 3 Oxygen content, solid reactant content and temperature at year 1 in a heap with permeability $K = 10^{-9}m^2$ and particle size $a = 0.005m$.

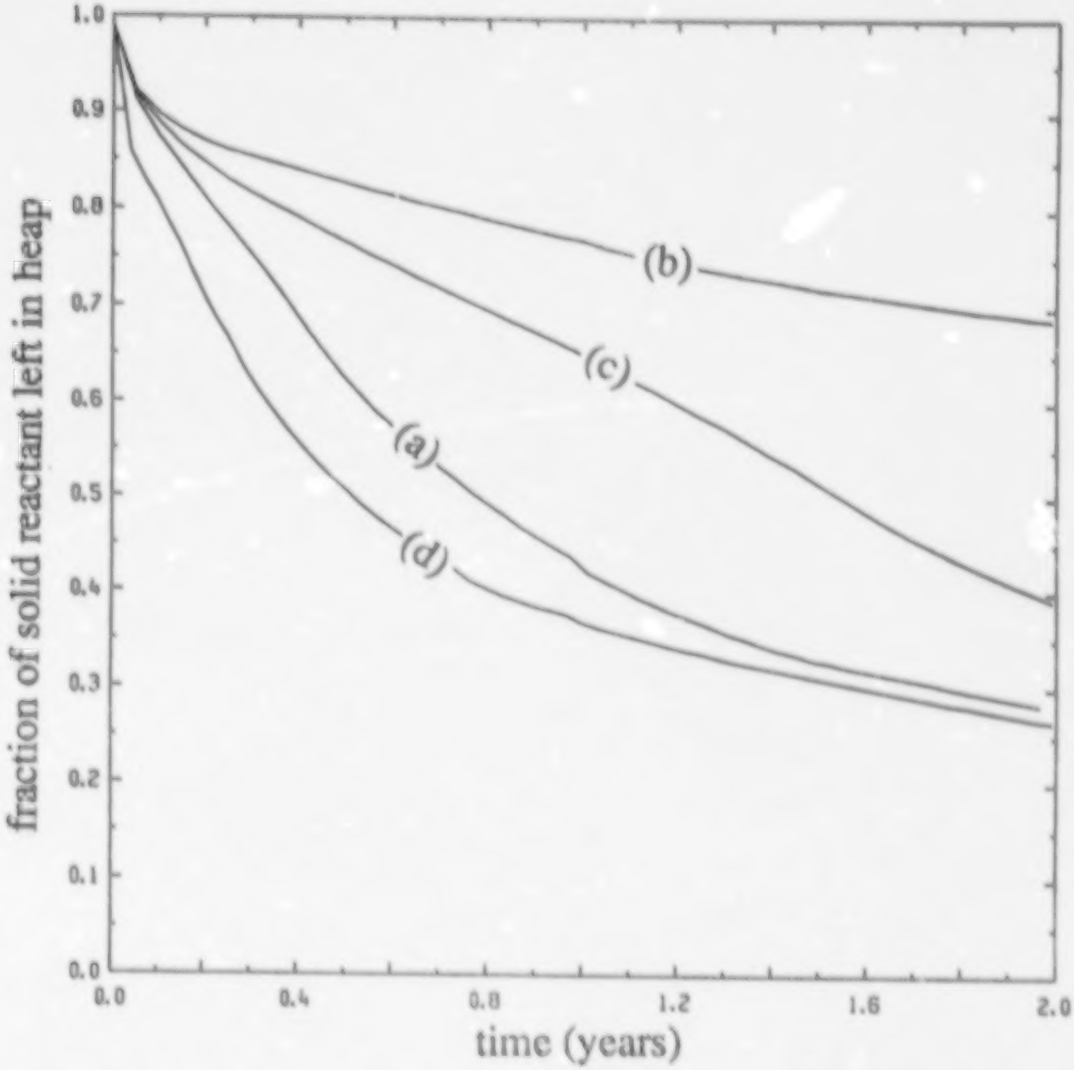

Fig. 4 Fraction of solid reactant left in the heap as a function of time. The cylindrical heap has a height of 20m, radius 20m and permeability $K = 10^{-9}m^2$. The 4 curves correspond to; (a) no irrigation and particle size a = 0.005*m*, (b) irrigation rate 0.01 *m/h* and a = 0.005*m*, (c) irrigation rate 0.001 *m/h* and a = 0.005*m*, (d) no irrigation and a smaller particle size a = 0.002*m*.

PILOT PRACTICE OF CONTINUOUS BIOLEACHING OF A GOLD REFRACTORY SULFIDE CONCENTRATE WITH A HIGH AS CONTENT

Dominique Morin[1] *& Pierre Ollivier*[1]

[1] *BRGM — Mineral Processing Department Avenue de Concyr, BP 6009,*
45060 Orléans Cédex — FRANCE

ABSTRACT

Two tests of continuous bioleaching by a mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* of gold refractory sulfide concentrate with a high arsenopyrite content have been carried out. In both cases mechanically agitated overflowing reactors have been used.

The first laboratory scale unit used is a series of four reactors agitated by two propellers with six 45° angled blades and representing a 100 L total capacity. The nominal solid flowrate was 2 kg/day. The total residence time was about 7 days. The test was run during 5 months. Back-mixing of bacteria was operated by recycling a part of the residual solution of bioleaching.

The second pilot scale unit is made up of four 800 L identical reactors agitated by two three-pitched-blade propellers and a Rushton-like turbine at the bottom to ensure dispersion of air. The solid flowrate is 100 kg/day. The total residence time is about 5 days in the test described here. This unit is fully equipped with BRGM proprietary systems for on-stream analysis of the bioleaching solution and data acquisition. The test lasted one month.

In both cases the temperature of the medium was maintained at about 35°C.

It has been established that in spite of a difference of content in arsenopyrite, ie, 40% and 50% respectively, the kinetics of decomposition of arsenopyrite is similar in both tests. Oxidation of arsenopyrite reaches 80% after a residence time of about 120h.

Even after a longer retention time pyrite remains unaffected by bioleaching. This result is interpreted as a consequence of the abundance of arsenite in solution which would act as a growth inhibitor and buffer of electrochemical potential.

A linear relationship exists between arsenopyrite decomposition and gold recovery. 80% of oxidation of FeAsS leads to 92% recovery of gold by cyanidation against 71 to 75% before bioleaching in both tests.

EXPÉRIENCE PILOTE DE LIXIVIATION BIOLOGIQUE EN CONTINU D'UN CONCENTRÉ AVRIFÈRE RÉFRACTAIRE SULFUREUX À TENEUR ÉLEVÉE EN AS.

D. Morin et P. Ollivier

B.R.G.M. – Département du Traitement des Minéraux
Orléans Cédex, FRANCE

RÉSUMÉ

On a mené deux essais de lixiviation biologique continue par une culture mixte composée de *Thiobacillus ferrooxidans* et *Thiobacillus thiooxidans* d'un concentré avrifère réfractaire sulfureux à teneur élevée en arsénopyrite. Dans les deux cas, on a utilisé des réacteurs de décharge à agitation mécanique.

Le premier appareil à l'échelle du laboratoire était constitué de quatre réacteurs agités par deux hélices à six pales inclinées à 45°, pour une capacité totale de 100 L. Le débit nominal de matières solides était de 2 kg/jour. Le temps de séjour total fut d'environ 7 jours. L'essai a été mené sur une période de 5 mois. On a procédé au remélangeage des bactéries en recyclant une partie de la solution résiduelle de lixiviation biologique.

Le deuxième appareil, à l'échelle de l'installation pilote, était constitué de quatre réacteurs identiques de 800 L. et agités par deux hélices à trois pales inclinées et d'une turbine du genre Rushton disposée au bas pour assurer la dispersion d'air. Le débit de matières solides était de 100 kg/jour. Le temps de séjour total a été d'environ 5 jours pour le présent essai. L'appareil était muni de systèmes du B.R.G.M. pour l'analyse en production de la solution de lixiviation biologique et pour la cueillette de données. L'essai a été mené sur une période d'un mois. Au cours des deux essais, la température du milieu a été maintenue à environ 35°C.

On a pu établir que, malgré une différence de la teneur en arsénopyrite, soit 40 % et 50 % respectivement, la cinétique de la décomposition de l'arsénopyrite était identique dans les deux cas. L'oxydation de l'arsénopyrite a atteint 80 % après un temps de séjour d'environ 120 heures.

Après un temps de séjour plus long, la pyrite n'était plus affectée par la lixiviation biologique. Ce résultat peut être interprété comme étant une conséquence de l'abondance d'arsénite dans la solution et qui pourrait agir comme inhibiteur de croissance et amortisseur de potentiel électrochimique.

Il existe une relation linéaire entre la décomposition de l'arsénopyrite et la récupération de l'or. Une oxydation de 80 % du FeAsS permet une récupération de 92 % de l'or par cyanuration par rapport à 71 % et 75 % avant la lixiviation biologique dans les deux essais.

INTRODUCTION

Several procedures exist for carrying out bioleaching tests at laboratory scale.

Atkins and Pooley (1983) have shown that at laboratory scale the comparison between shake-flask, stirred tank, pachuca reactor and static column techniques was unfavourable to the stirred reactor. The tests were carried out in the conditions of a rather long-term biotreatment. However in the case of fast-kinetic bioleaching as required for pretreatment of a gold refractory sulfide concentrate the mechanically agitated reactor is in fact the main technique to have been retained for industrial application. Its principal advantage consists in its ability to maintain homogeneity for the gas-liquid-solid medium with many different designs and configurations providing great flexibility relative to power consumption. However one type of agitator needs to be tested gradually in size-steps to optimize its efficiency and power consumption.

Furthermore beyond the bench-scale tests the development of a new process flowsheet involves pilot-plant testing to assess the validity of scale-up procedures.

In this context a small continuous pilot unit comprising four 800 L agitated tanks has been built and operated. The first aim of the pilot campaign was to study the treatment by bioleaching of a gold refractory sulfide concentrate mainly as arsenopyrite. The second aim was testing an agitator scale-up rules of which are known with confidence. The third aim was testing equipment for on-stream analysis and computerized data acquisition.

This campaign was undertaken after adaptation and testing of a mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* on the substrate during two years of continuous bioleaching in a 100 L agitated bench-scale unit.

Arsenic is solubilised from arsenopyrite as As^{III} and As^{V} (Panin *et al.*, 1985), and iron as Fe^{II} and Fe^{III}. The main reactions involved in enhancement by bacteria metabolism are the following:

$$2FeAsS + 5.50_2 + 3H_20 \rightarrow 2H_3As0_3 + 2FeS0_4 \tag{1}$$
$$2FeAsS + 6.50_2 + 3H_20 \rightarrow 2H_3As0_4 + 2FeS0_4 \tag{2}$$
$$2FeS0_4 + H_2S0_4 + 0.50_2 \rightarrow Fe_2(S0_4)_3 + H_20 \tag{3}$$
$$2S^{\circ} + 2H_2O + 3O_2 \rightarrow 2H_2SO_4 \tag{4}$$

Arsenious acid, H_3As0_3, produced through reaction (1) is also partially oxidized in arsenic acid, H_3As0_4.

A high proportion of the products of these reactions precipitates as follows (Livesey-Goldblatt *et al.*, 1983):

$$Fe_2(So_4)_3 + 2H_3As0_4 \rightarrow 2FeAs0_4 + 3H_2S0_4 \tag{5}$$
$$Fe_2(S0_4)_3 + 6H_20 \rightarrow 2Fe(0H)_3 + 3H_2S0_4 \tag{6}$$
$$Fe(0H)_3 + 3H_2S0_4 \rightarrow Fe(0H)(S0_4) + 2H_20 \tag{7}$$

With the increasing decomposition of arsenopyrite the inhibitory effect of species in solution on bacteria growth appears. However even in the case of a high as content concentrate the attack of the arsenopyrite can be complete with a specifically adapted strain. Also it must be noted that simultaneously pyrite may remain unaffected (Morin *et al.*, 1989).

This paper describes the 3.2 m^3 pilot plant and gives the data collected for a one-month period compared to those obtained with the 100 L continuous laboratory scale unit over a 5-month period.

MATERIALS AND EQUIPMENT

The inoculum

The inoculum used was obtained by enrichment of cultures from slurries sampled in sulfide ore mines. The basal enrichment medium contains 3.7 g/l ferrous $(NH_4)_2SO_4$, 0.8 g/l, 85% H_3PO_4, 0.5 g/l KOH, 0.5 g/l $MgSO_4$, $7H_2O$, 3% sulfide compound and 9 g/l ferrous ion as ferrous sulfate. In order to obtain strains adapted to arsenopyrite treatment, the inoculum was cultivated in successive basal media in which ferrous ions have been progressively replaced by sulfide substrate up to 10% (wt/vol).

The cultures were made in air-lift aerated and agitated tubes. The temperature was maintained at 35°C by a thermostated bath.

The inoculum obtained was cultivated first in a 1-litre batch reactor. Afterwards the biomass obtained was used to inoculate the 100 L continuous laboratory pilot that has been in operation for three years.

The strain characterisation has shown that the inoculum is made up of a mixed culture of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* (Collinet and Morin, 1989).

The arsenopyrite concentrate

During the 5-month period of bioleaching in the 100 litre bench-scale unit reported here the arsenopyrite concentrate from a French mine typically contained 35.5% iron, 19.9% arsenic and 27.7% sulfur as sulfides. These data can be used to calculate the approximate proportions of the principal sulfides, which gives, on the basis of their simplest formula: 40% FeAsS, 28% FeS2 and 14% FeS.

For the reported pilot test the concentrate composition contained more arsenopyrite, with 32.6% Fe, 22.85% As and 24.8% S =, which gives 50% FeAsS, 28% FeS2 and 7.8% FeS.

Gold grade was 42.85 g/t in the first case and 15 g/t in the second.

Optical microscope observation of polished sections of the concentrates shows that there are few mixed FeAsS-FeS2 grains. Gold occurs largely as native fine grains but 30 to 40% is trapped in other mineral components.

The grain-size distribution of both concentrates were similar with 95% of particles smaller than 64 μm.

The laboratory unit

The laboratory unit is a cascade of four mechanically agitated PVC reactors double-jacketed for thermostating at 35°C. The first reactor has a volume of 40 L, the others 20 L.

Each of the four tanks is agitated by 2 propellers with 6 blades angled at 45° which are fixed to a shaft rotating at a 450 r.p.m. speed. Air is injected with a flowrate of 0.35 L of air per litre of pulp per minute under the lowest propeller.

At a 2 kg/day nominal dry solid flowrate and a solid ration of 12% (wt/wt) the residence time of pulp is close to 7 days for the whole unit.

About 13% of the final bioleaching solution is recycled. This enables the slight natural basicity of the fresh concentrate to be neutralized and a back-mixing of bacteria to be effected.

The nutrients are the same as in the basal enrichment medium at the same concentrations but without ferrous iron.

The pilot unit

The pilot unit (see photo 1) is a series of 4 identical overflowing double-jacketed PVC reactors. The volume of one reactor is 800 litres.

The temperature of the medium is maintained at 35°C.

The pulp agitation system has been designed and manufactured by Robin Industries (FRANCE). The pulp suspension is ensured by two three-pitched-blade propellers and the aeration by a bottom Rushton-like turban with air injection under its center. The agitation shaft rotates at a 150 r.p.m. speed. The air flowrate for the whole unit is 0.2 m^3 of air/m^3 of pulp/min. In the same working conditions of pulp as for the bench-scale unit but for a 100 kg/day solid flowrate the retention time is about 4 days nominally.

The pulp used for a feeding of two or three days is prepared in a 2.5 m^3 agitated tank which lies on weight gauges. The residue of bioleaching is recovered in a 10 m^3 tank which is weighed in the same way as the feed tank.

Data acquisition system on the pilot unit

The pilot unit is equipped with an automatic system of data-acquisition (figure 1) consisting of:

- a filtering system called PIRANA* (Pulp Instrument for Regulation and ANAlysis) which removes clear liquid from pulp extracted successively from the feed tank and each reactor by a peristaltic pump and measures the pH, Eh, dissolved oxygen and temperature of the liquid.
- a SYRANO^R X-ray fluorescence analyser which measures iron and arsenic concentrations in the filtered solution from PIRANA.
- a pneumatic robot for sampling and distribution of pulp and liquid fractions.

Pulp samples can be taken by pneumatic samplers on each analysed back flow.

All data from PIRANA, SYRANO, weight gauges and air mass flowmeters converted into analogic electrical signals are collected and periodically averaged by a PC using a specific software. This software also triggers alarms in case of defaults in air supply and data acquisition systems.

Laboratory analyses

Iron and arsenic precipitated in the bioleaching medium are titrated on filtered solids from pulp by digestion in weakly acid solutions at 40°-50°C.

Concentrations in iron and arsenic in solutions and solids are measured by XRF (SYRANO). Arsenite (As^{III}) is determined by polarography and this concentration subtracted from the total As content gives the arsenate (As^{V}) concentration.

Gold liberation is evaluated by cyanidation on filtered bioresidue. The bioresidue is repulped in water, neutralised at pH 10.5 by slaked lime and cyanided with 10 g/l of NaCN.

Free bacteria in solution are counted with an optical microscope (x 400) using a Thoma counting cell.

RESULTS AND DISCUSSION

Variation of the parameters measured in the bioleach solutions

For a given theoretical residence time, ie, for each reactor, the daily measurements of pH, Eh, [Fe], [As] and concentration of bacteria in solution have been averaged for the overall duration of treatment. The treatment lasted 150 days for the 100 L unit and 31 days for the 3.2m^3 unit. The residence time is evaluated taking into account the real capacity of the reactors and the average pulp feed rate.

The variation of pH with residence time for both units is shown on figure 2.

A certain similarity or rather complementarity of the two curves can be seen. The lower pH for the laboratory unit treatment is probably only due to the recycling of acid solution.

The variation in Eh, see figure 3, shows similar features. The recycling of oxidising solution would lead to an increase of potential throughout the reactors of the laboratory unit compared to that in the pilot unit.

The continuous seeding of bacteria into the first reactor of the laboratory unit does not cause a rise in the bacteria population as compared with that in the pilot unit (see figure 4). The growth could even have been reduced by the recycling as slows down and finally levels off at a lower rate. The population reaches a maximum as quickly as it does in the second reactor and is stable later on. The maximum value of concentration of bacteria is 1 to 2 x 10^9 bacteria per ml.

Figures 5 and 6 show that, taking into account the quantities of iron and arsenic recycled for the laboratory unit, almost the same amount of iron appears in solution in both treatments and slightly more arsenic in the pilot treatment. Nevertheless the shapes of the curves for both treatments are different. At the pilot scale the kinetics are initially faster than at the laboratory scale, but then tends to stabilized. In the laboratory scale treatment the dissolution of iron and arsenic varies almost linearly. Again this appreciable difference is explained as a consequence of the recycling of acid solution which leaves less iron and arsenic recipitated (mainly) as ferric arsenate.

Titrations of arsenite (As^{III}) and arsenate (As^{V}) in the solutions of each reactor of the 3.2 m^3 unit have shown that dissolved arsenic is essentially present as arsenite (figure 7). Only in the last reactor does the arsenate concentration reach a significant level. After a residence time of 100 hours the acidity may be sufficiently high to impede the precipitation of arsenate.

The abundance of arsenite is possibly the main cause of limited bacterial growth in the medium as As^{III} is the most toxic dissolved element (Groudev, 1988, and Collinet and Morin, 1989). This must also be taken into consideration for the detoxification of the residual solutions since it is desirable to eliminate the arsenic as (ferric) arsenate which is the most stable solid As residue. This suggests that an additional step, of oxidation of the residual solutions, should be included in the process flowsheet.

In spite of the difference in arsenopyrite content, 40% and 50% for the concentrates treated respectively in the laboratory and pilot units, the overall oxidation of FeAsS, taking into account the quantities of iron and arsenic in solution and precipitated, follows almost the same kinetics in both cases as shown on figure 8. The kinetics of the pilot scale treatment is only slightly faster.

The decomposition kinetics of arsenopyrite seems to be controlled by chemical or biochemical phenomenon on which the size of the equipment has no influence, provided that sufficient homogeneous aeration and homogeneous suspension of solids are maintained. The back-mixing of bacteria does not improve the rate of attack.

Moreover, it has been observed that pyrite is not affected by the bioleaching (figure 9), only arsenopyrite and pyrrhotite are decomposed. The arsenopyrite remaining after the longest residence time, ie, 168 hours, is probably trapped in another mineral, possibly pyrite, as a mixed grain.

It is probably typical of the bioleaching of arsenopyrite-rich concentrate with a minor pyrite content that the dissolution of a large quantity of arsenic in solution mainly as arsenite leads to a relative low acidity of the medium, a low oxidation potential and finally the selectivity of attack.

A test of re-bioleaching of the pyritic residue of the first bioleaching after having separated liquid and solid has shown that the residual pyrite could be dissolved. Thus pyrite in this case is not naturally refractory to bioleaching. It has also been observed that, with the same bacterial strains, in concentrates where pyrite is the chief mineral and arsenopyrite the minor one selectivity no longer occurs.

Recovery of gold

As can be seen on figure 10 gold recovery by cyanidation and the rate of oxidation of arsenopyrite show linear correlation. Only two results are at present available for the test with the 3.2 m^3 unit. One is the amount of free gold that can be extracted before bioleaching, which reaches 71.1%, the other is the recovery of gold after complete treatment of the concentrate in the four reactors (for a residence time of 118 hours) which is 92.9%. These two results tend to confirm the variation of gold liberation according to the decomposition of FeAsS deduced from the test with the 100 L unit.

The linear relationship between the arsenopyrite oxidation rate and gold recovery seems to indicate that refractory gold recovered is finely and homogeneously distributed in FeAsS.

Extrapolation of the relation between Au recovery and the percentage of oxidation of FeAsS suggests that refractory gold occurs not only in arsenopyrite but probably also in at least one other mineral.

CONCLUSION

The bioleaching of concentrate with arsenopyrite as the main mineral and pyrite as a minor mineral leaves pyrite unaffected, probably because of the conditions of ionic equilibrium in solution. The abundance of arsenopyrite as the first source of energy supply for the bacteria maintains a large proportion of the oxidized arsenic as arsenite. Arsenite would act as an inhibiting agent for the growth of bacteria and also as a reducing or buffering agent for solution potential. These two phenomena preclude the further attack of pyrite.

Provided that homogeneity of gas dispersion and pulp is maintained the decomposition kinetics of arsenopyrite remain the same in different sized units, under similar treatment conditions. In this study, both for a 100 L unit and for a 3.2 m^3 unit (respectively at 2 kg/day and 100 kg/day concentrate flowrates) arsenopyrite decomposition reached 80% after a residence time of about 120h.

Back-mixing of bacteria has no positive effect on the bioleaching.

A linear relationship exists between arsenopyrite decomposition and gold recovery and 80% of oxidation of FeAsS leads to a 92% recovery of gold by cyanidation against 71 to 75% before bioleaching in both tests.

The shearing effect caused by the motion of the Rushton-like turbine used in the 3.2 m^3 unit does not affect the growth of bacteria. The complete agitator tested is suitable for this type of treatment and will be optimized with respect to the power consumption and scaled up.

The BRGM proprietary equipment for direct analyses of bioleaching solution and the data acquisition system enable manual operations to be substantially reduced and could be used for other comparable situations.

Additional results, concerning in particular the further stages of treatment, ie, elimination of arsenic and cyanidation, will be communicated later.

The analysis of all the data collected will lead to the decision to continue the study at a larger scale. The next stage would be to operate an industrial pilot scale unit with a capacity of 5 tons of sulfide concentrate per day.

ACKNOWLEDGEMENTS

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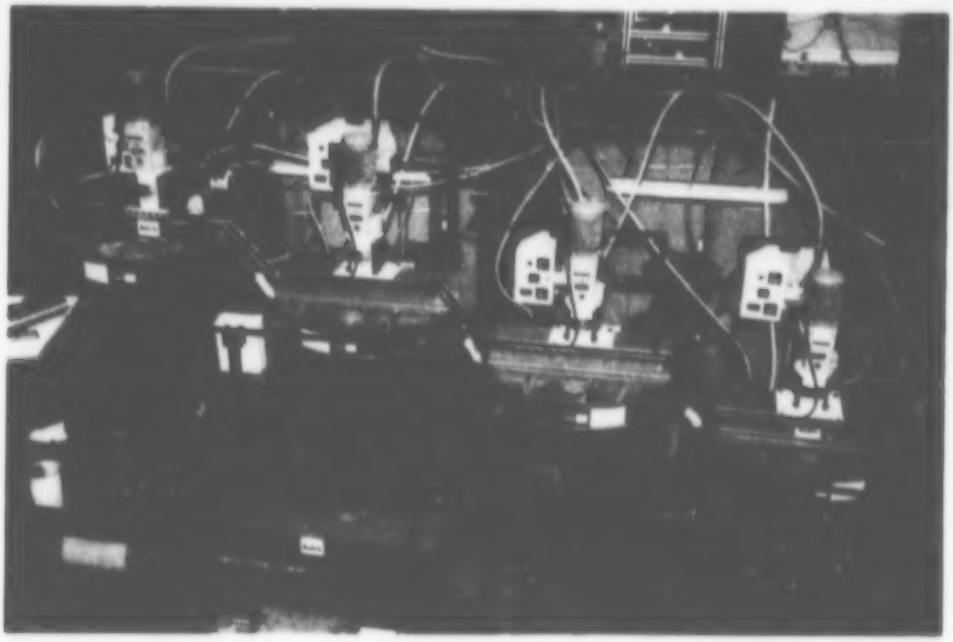

Photo 1 View of the cascade of reactors of the 3.2 m^3 unit.

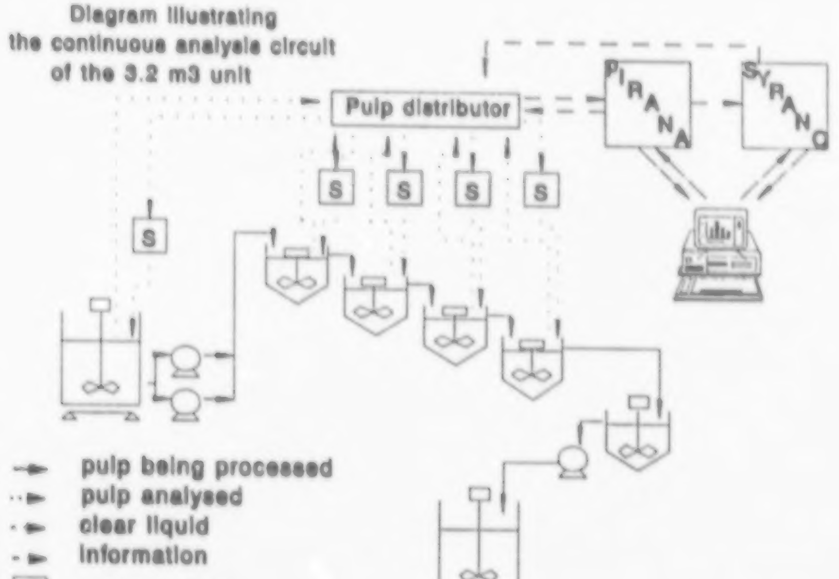
Diagram illustrating
the continuous analysis circuit
of the 3.2 m3 unit
Pulp distributor
PIRANA
SYRANO
S
pulp being processed
pulp analysed
clear liquid
information
S pulp samplers

Fig. 1 Diagram illustrating the continuous analysis circuit.

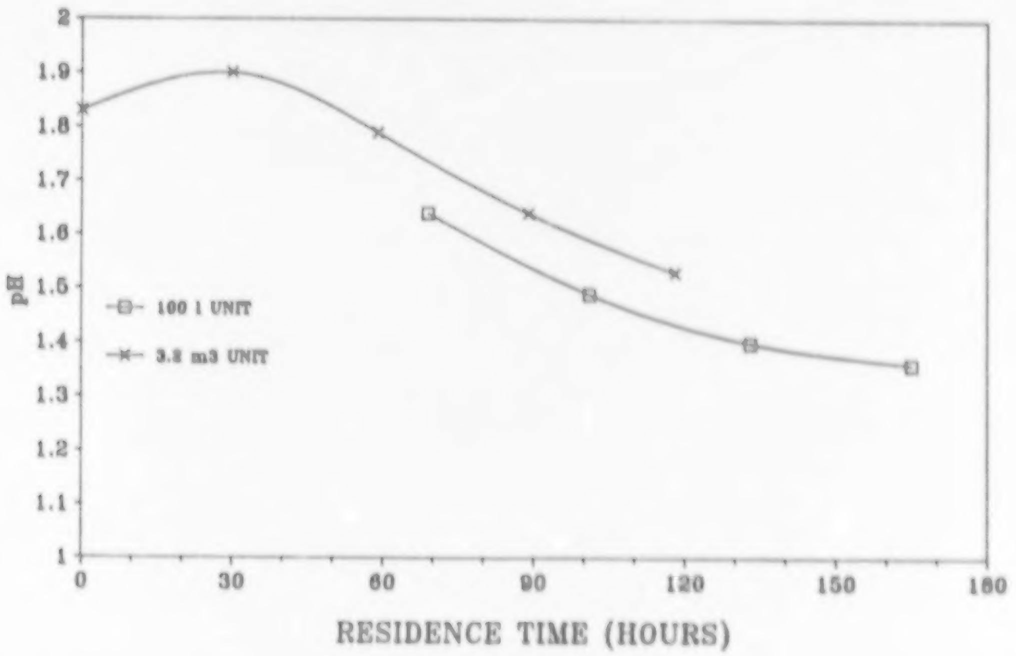

Fig. 2 pH vs residence time for the 100 L and 3.2 m^3 tests.

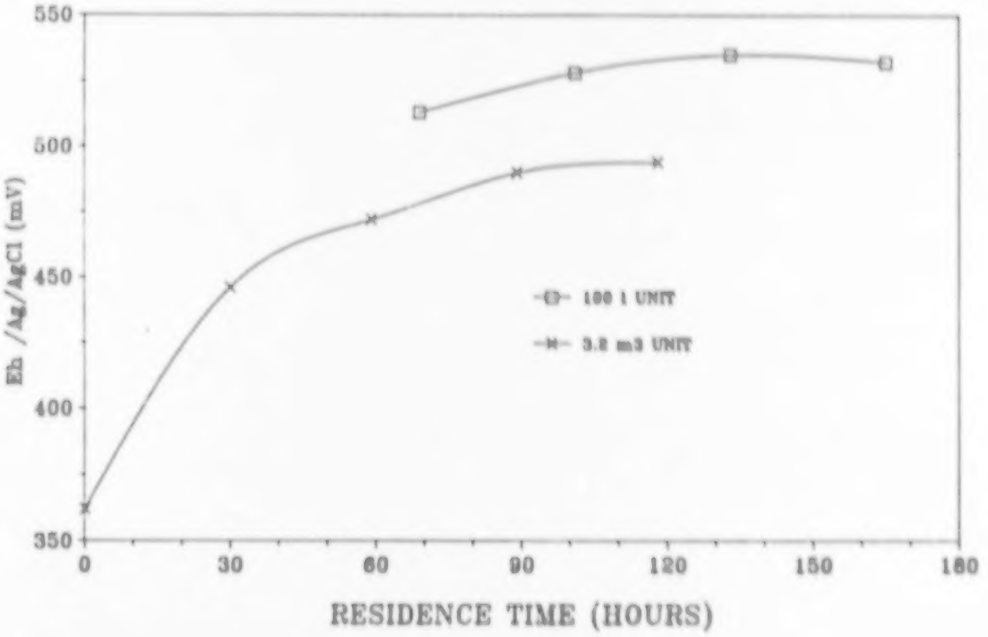

Fig. 3 Eh vs residence time for the 100 L and 3.2 m^3 tests.

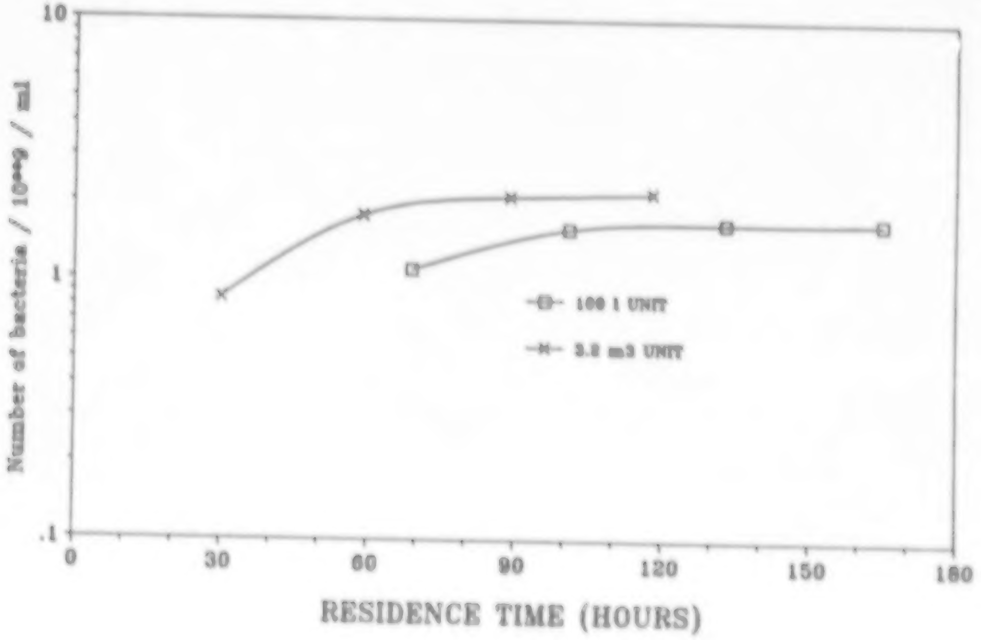

Fig. 4 Concentration of bacteria in solution vs residence time for the 100 L and 3.2 m³ tests.

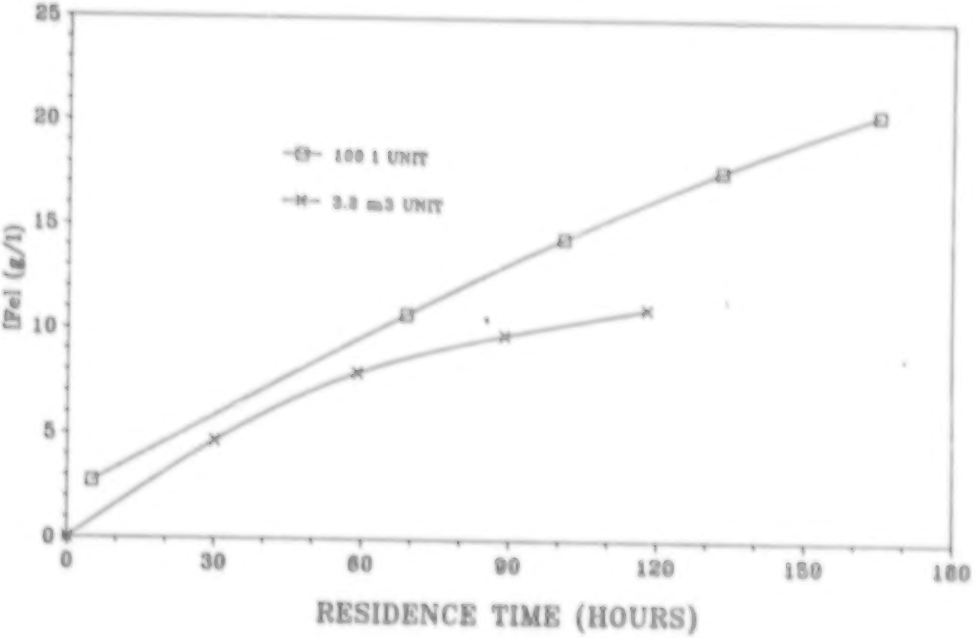

Fig. 5 Concentration of Fe in solution vs residence time for the 100 L and 3.2 m³ tests.

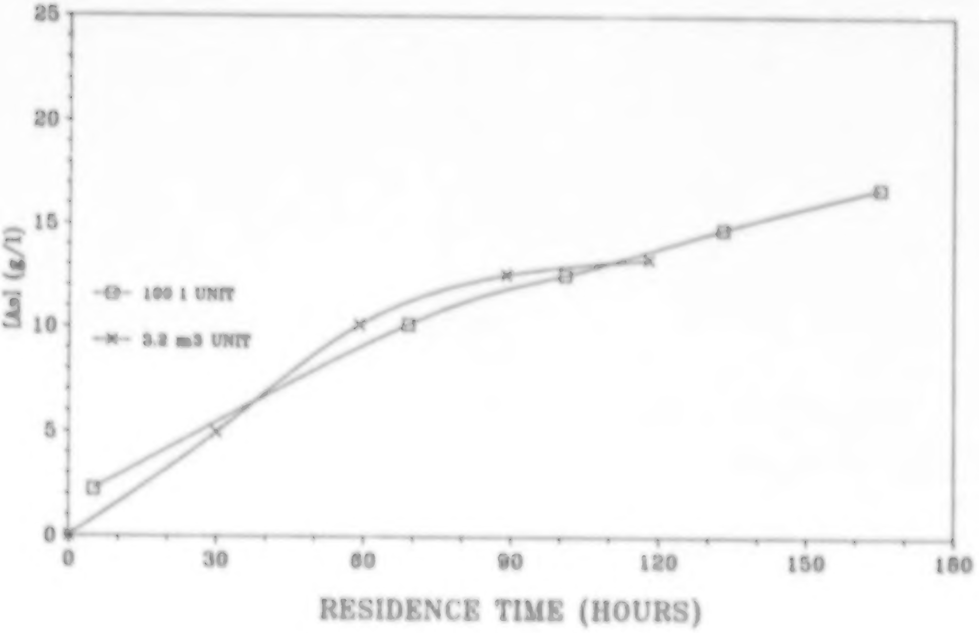

Fig. 6 Concentration of As in solution vs residence time for the 100 L and 3.2 m³ tests.

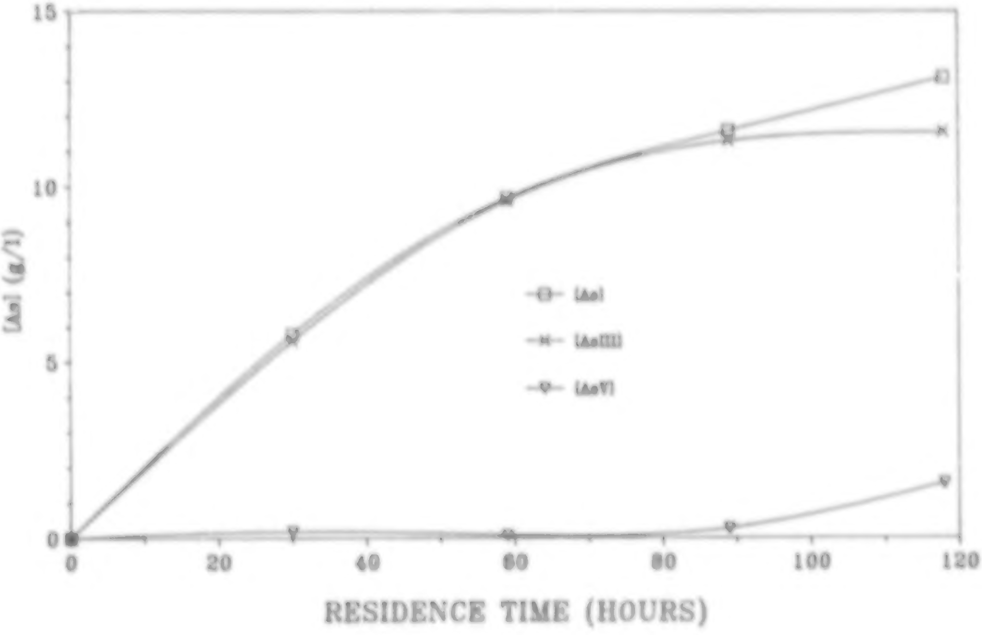

Fig. 7 As^{III}, As^{V} and total As concentrations vs residence time for the 100 L and 3.2 m³ tests.

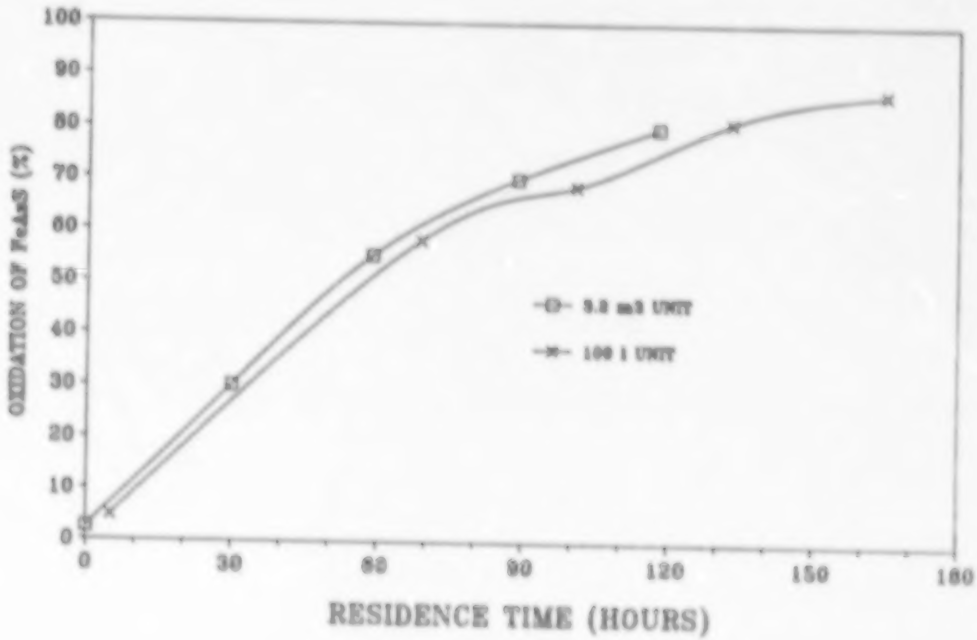

Fig. 8 Arsenopyrite oxidation vs residence time for the 100 L and 3.2 m³ tests.

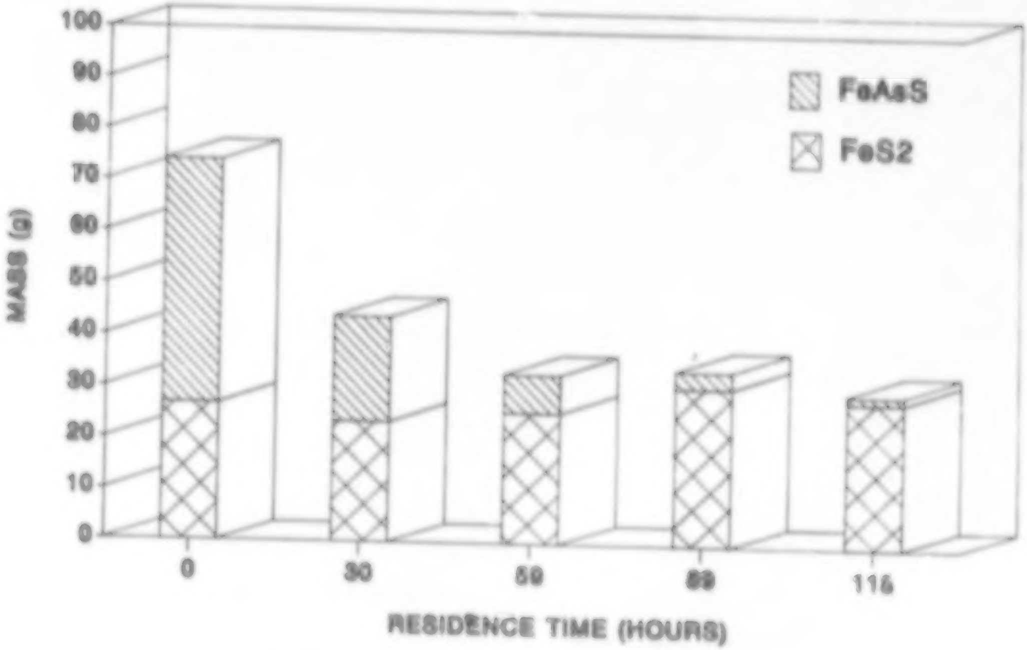

Fig. 9 Masses of FeAsS and FeS_2 for 100 g of initial concentrate vs residence time.

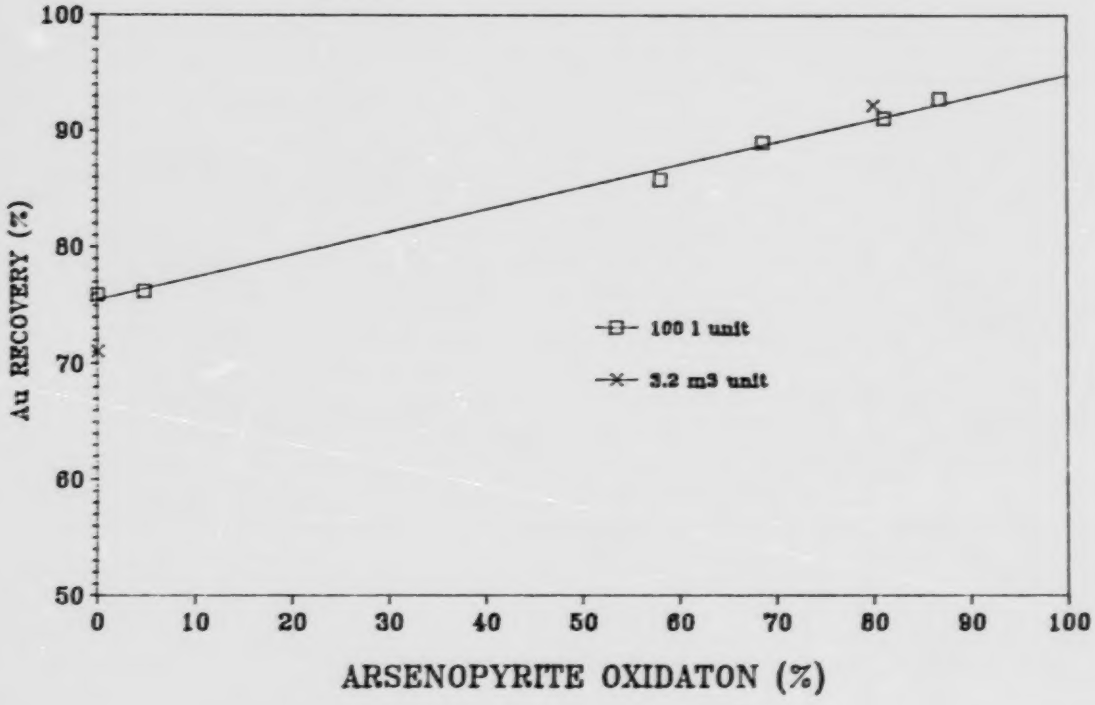

Fig. 10 Au recovery by cyanidation vs arsenopyrite oxidation.

SCALE-UP OF LEACHING OF MALANJKHAND COPPER ORES-A CASE STUDY

A.D. AGATE, K.M. PAKNIKAR and N.J. KHINVASARA

Department of Microbiology, MACS Research Institute, Law College Road, Pune – 411 004, INDIA

ABSTRACT

The Malanjkhand copper deposit, located in central India, has large quantities of lean sulfide and oxidized ore comprising mainly chalcopyrite with some malachite and azurite. In order to develop a bioleaching process for commercial extraction of copper from lean sulfide ore, we attempted a scale-up from bench scale (100 g) to 8.2 ton level at the mine site. A culture of *T. ferrooxidans* MCM B-7 from MACS culture collection was used for all the experiments. The final scale-up stage was 5 m high concrete column. A total of 8.2 tons of lean sulfide ore (size: +1" = 25%; -1" = 75%) having a copper content of 0.33% was leached over a period of 180 days with solution application rate of approximately 10 L/m^2/h. Prior to inoculation of *T. ferrooxidans*, the gangue material was neutralized by sulfuric acid. The column was given rest intermittently. Over the 180-day period copper extraction was 19%, recovered as cement copper. The total amount of sulfuric acid consumed was 0.63% w/w. Taking into consideration the above results and labour and other costs, feasible commercial scale leaching is projected for malanjkhand copper ore.

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LIXIVIATION À GRANDE ÉCHELLE DU MINERAI DE CUIVRE DE MALANJKHAND — UNE ÉTUDE DE CAS

A.D. Agate

Département de Microbiologie, M.A.C.S. Institut de Recherche, Pune, INDE

RÉSUMÉ

Les dépôts de cuivre de Malanjkhand au centre de l'Inde renferment de grandes quantités de minerai oxydé et à faible teneur en sulfure, composé principalement de chalcopyrite et de certaines quantités de malachite et d'azurite. Afin de mettre au point un procédé de lixiviation biologique pour l'extraction commerciale du cuivre contenu dans le minerai, à faible teneur en soufre, nous avons tenté d'élever les proportions étudiées à l'échelle du laboratoire (100 g) à 8.2 tonnes au site de la mine. Dans toutes les expériences, nous avons utilisé une culture *Thiobacillus ferrooxidans* MCM B-7 provenant de la banque de cultures du M.A.C.S. L'expérience finale à grande échelle comprenait la lixiviation du minerai dans une colonne de béton de 5 m de hauteur. Une masse totale de 8.2 tonnes de minerai à faible teneur en soufre, (calibre : plus de 1" = 25 %; moins de 1" = 75 %) et renfermant 0.33 % de cuivre a été lixiviée sur une période de 180 jours, l'application de la solution se faisant sous un débit approximatif de 10 L/m^2/h. Avant l'inoculation de *T. ferrooxidans*, la matière en gangue fut neutralisée au moyen d'acide sulfurique. On laissait reposer la colonne par intermittence. À la fin de la période de 180 jours, on avait extrait 19% de cuivre sous forme de cuivre de cémentation. La quantité totale d'acide sulfurique utilisée fut de 0.63% (en poids). Si on tient compte des résultats mentionnés et des coûts de main-d'oeuvre et autres, il est possible d'apprécier la rentabilité à l'échelle commerciale de la lixiviation du minerai de cuivre de Malanjkhand.

INTRODUCTION

Copper production in India, which has grown steadily over the years, is managed by a Public Sector undertaking, the Hindustan Copper Ltd. The present yearly demand for copper in India is estimated to be around 116,000 tons, of which only 30% is met by domestic production and the balance of 70% is imported. In an urgent endeavour to improve domestic productivity, Hindustan Copper Ltd. has embarked on various technological innovations and expansion plans. The commissioning of the Malanjkhand Copper Project (MCP) located in Central India in the State of Madhya Pradesh is viewed as a major step in this direction. The Malanjkhand deposit consists mainly of chalcopyrite with some malachite and azurite. The ore material is mined by open cast method, and huge quantities of lean sulfide ore, which is not fed to the concentrator plant, is generated. It is reported that in the course of operating the MCP open pit mines, about 0.7 million tons of lean sulfide waste (average grade 0.3% copper) have been produced. The total quantity of lean sulfide ore available at MCP is estimated at 23 million tons.

Although biohydrometallurgical processes for the extraction of copper are being used in many parts of the world, large-scale application of such extraction processes in India is virtually non-existent. With a view to increasing copper production from the existing resources, this technology is proposed for use at Malanjkhand, to recover copper from the waste lean sulfide ore. The possibility of bioleaching of Malanjkhand ores has been studied earlier at MACS Institute (Agate and Khinvasara, 1986). During the present studies the work on bioleaching of Malanjkhand lean sulfide ores has been scaled-up by increments, with the largest effort to date at the mine site.

MATERIALS AND METHODS

Culture

A culture of *Thiobacillus ferrooxidans* MCM B-7 from the MACS Collection of Microorganisms was used for all the experiments. This culture was isolated indigenously from Malanjkhand Mines by enrichment culture technique (Paknikar and Agate, 1987). The culture was routinely maintained in liquid 9K medium of Silverman and Lundgren (1959). The culture was simultaneously preserved in 9K basal salts medium containing Malanjkhand lean sulfide ore for leaching experiments. For mine site experiments, the culture was transported to Malanjkhand in the preserved form, using the method described by Gupta and Agate (1986).

Ore

A bulk sample of lean sulfide ore was collected from the sorted out material at MCP. This ore sample was designated A I-53. The ore sample was subjected to chemical and mineralogical analysis by routine methods. Before carrying out the leaching experiments, preliminary evaluation tests for determination of pH, acid consumption and acid production potential were carried out using the methods of Doemel and Brock (1971), Bierhaus *et al* (1983) and Bruynesteyn and Hackl (1985), respectively. At the mines, for column leaching experiments, system factors (porosity, permeability and rate of recirculation) and size analysis of feed material were evaluated in addition to the preliminary tests mentioned above.

LEACHING PROCEDURES

At the laboratory, the following methods were used:

Flask Leaching

Flask leaching experiments were carried out in 250 ml Erlenmeyer flasks containing 10 ml of active, well-grown culture of *T. ferrooxidans* MCM B-7, 90 ml iron-free 9K medium at pH 2.0, and 10 g of finely ground (-52 mesh) Malanjkhand ore AI-53. Prior to inoculation, the flasks were sterilized by autoclaving. In the control flasks, 10 ml of 2% solution of thymol in methanol was added as a bacteriostatic agent, instead of the inoculum. The flasks were incubated at room temperature (28 + 2°C) on a rotary shaker with an agitation rate of 250 rpm.

Air-lift Percolator Leaching

A battery of air-lift percolators (100 cm height X 9 cm diameter) was used in this study. The percolators had a retention volume of 2 liters and the air-lift tube at the side of the percolator had an internal diameter of 8 mm. Each percolator was charged with 2.5 kg Malanjkhand ore (+ ½"; -1") and 1800 ml of iron-free 9K medium and 200 ml inoculum (10% v/v) of *T. ferrooxidans* MCM B-7.

PVC Column Leaching

The leach columns used in these studies were made of rigid PVC (polyvinyl chloride) material, capable of withstanding working pressure and acidity. Each column was 150 cm high with a 30 cm inner diameter, and was closed at one end by a PVC end-cap. The columns were placed on a steel stand, and a 100L plastic vat was placed below each column. All of the vats had an air-lift arrangement for recirculation of leached liquor which percolated from the column. The columns were charged with 75 kg of Malanjkhand ore Al-53 of size +½"; -1" and 75 liters iron-free 9K medium containing 10% v/v *T. ferrooxidans* MCM B-7 as inoculum and the medium was recirculated.

Mine Site Leaching in a Concrete Column

At the mine site, 5 m high column, constructed by joining two concrete pipes of 1.2 m diameter, was used for carrying out the leaching experiments (Fig. 1). The column was made leakproof by cementing at the joints and acid-proofed by applying a coat of acid-proof epoxy paintto the inner surface.

(1) Selection of ore: The ore material selected for the experiment was collected from the waste ore dumps near the mine. Approximately, 16 tons of this material were collected and the material as manually sorted to remove quartz, oxidized ore and high grade sulfide ore. The low grade and the high grade sulfide material were then crushed separately in a jaw crusher (1" opening) and then mixed. Samples were removed after coning and quartering to arrive at the desired grade. After several steps of blending and sampling, an 8.5 ton ore heap was obtained. The chemical and the size analysis of the ore was then carried out.

(2) Packing of the column: Before loading the crushed ore into the column, a filter bed was prepared at the bottom of the column. Its purpose was to prevent the washout of the fines and smaller ore particles from the column into the effluent tank. The bed consisted of a layer of river-bed gravel, topped with quartz particles (+1" to 2" size) and a thin layer of sand. The total thickness of the filter bed was about 18 cm. On top of the filter bed, about 300 kg of course ore particles (+1") was layered and then the crushed ore from the heap was packed into the column by hand, taking care to spread the ore evenly and prevent formation of cavities. Approximately 8190 kg of ore was load into the column, leaving a sufficient headspace for installation of the sprinkling system. The details of column loading could be summed up as follows:

Column height = 5 m
Column diameter = 1.2 m
Headspace height = 0.88 m
Height of ore column = 3.94 m
Weight of ore = 8190 kg
Volume of ore = 4.45 m^3
Bulk density = 1.84 g/ml

(3) pH stabilization: To stabilize the pH of the ore, around 2.0, initially 0.2% w/w sulfuric acid solution was applied manually at the top of the column and the solution was allowed to percolate through the ore. The column was then closed at the top with high density polyethylene (HDPE) sheet and the contents allowed to react over a period of 4 days. At the end of this period, the leachate was removed, measured and stored in HDPE tanks. To obtain a stable pH of 2.0 in the column, two additional steps of pH stabilization were conducted in a similar manner. The leachate obtained during these stages was also measured, and stored in HDPE tanks.

(4) Bacterial leaching: After the pH was stabilized around 2.0, the column was inoculated with *Thiobacillus ferrooxidans* culture. The ore-preserved *T. ferrooxidans* MCM B-7, grown in plastic vats in 9K medium and a fully grown culture inoculated at 10% v/v level in 700 litre acidified water of pH 2.0, was mechanically sprinkled over the ore in the column. This system of application facilitated even application on all sides and simultaneous aeration of the solution. The rate of application was maintained around 10 litres/m^2/h.

The experiment was closely monitored around the clock with sampling from influent and effluent solutions at 6-hour intervals. The samples were analysed for pH, Cu^{+2}, Fe^{+2}, Fe^{+3}, free acid and microbial count. The influent and effluent flow rate measurements were carried out hourly.

The pH of the system was maintained at 2.0 throughout the experiment by the addition of concentrated sulfuric acid as required. Evaporation losses during recirculation were replaced by addition of acidified tap water every 24 hours.

At intervals depending on the tenor of copper in the leachate, the leachate was removed and stored in HDPE tanks for subsequent copper recovery. The leaching cycle was then resumed using fresh acidified tap water (ca. 350 liters). Rest periods of 10-15 days were given to the column whenever copper extraction was found to reach a plateau. Test duration was 180 days.

(5) Cementation of copper: The leached liquor collected in HDPE tanks was put into a cone precipitator with aeration and manual agitation capabilities for cementation using scrap. The cemented copper was sun-dried in shallow trays.

RESULTS

Laboratory-Scale Experiments

The chemical and mineralogical analysis of the Malanjkhand lean sulfide ore Ai-53 is given in Table 1.

The results of the preliminary evaluation tests for determination of pH, acid consumption, acid production potential and effective porosity carried out on Malanjkhand lean sulfide ore were as follows:

pH = 8.3
Acid consumption by 100 g ore (-150 mesh) = 6 ml 5N Sulfuric acid
Acid production potential = positive
Effective porosity = 4.2%

Table 2 and Fig. 2 present the results obtained on flask, air-lift percolator and PVC column leaching studies. The copper extraction values were corrected with copper extracted from parallel controls.

In the flask leaching experiment, it was observed that the pH of the medium rose to almost 4.5 during the first two weeks of leaching, and had to be lowered to the initial pH of 2.0. After two weeks, the pH stabilized around 2.0. The curve for copper extraction in the flask leaching experiment showed an initial steep rise followed by flattening of the curve for a short period (up to two weeks) with a subsequent rise up to the 45th day. The copper extraction was then stabilised till the end of the experiment. Total copper extraction was 36.7% over a 60-day period.

In contrast to the flask leaching experiment, the pH of the ore in the air-lift percolator stabilized at pH 2.0 within a week and copper extraction continued to rise after an initial one-week lag period. Copper extracting stabilised after 90 days until the termination of the experiment at 120 days. Total copper extraction was 13.42%.

The results of the PVC column leaching experiment were comparable to those of air-lift percolator leaching. The system pH stabilized at 2.0 within a week and copper extraction progressed rapidly until the 120th day, after which the extraction stabilized and remained constant up to 180 days, despite a 10-day system rest period after 130 days. The total copper extracted was 14.32% in 180 days.

Mine-Site Leaching experiment

The chemical and size analysis of the ore AI-53 used for loading the 5 m column were as follows:

Chemical analysis:

Total copper = 0.33%
Acid-soluble copper = 0.02%
Sulfidic copper = 0.31%
Total iron = 2.35%

Size analysis:

+ 1" = 25%
= 1" to + ½ = 58%
-1½" to + ¼" = 8%
-¼" (mostly fines) = 9%

The effective porosity of bulk ore in the column was 4.2% and its sulfuric acid consumption was 0.18% - 0.3% w/w as determined by experimentation.

Stabilization of the column to pH 2.0 required 40 days period with several acid addition steps and a rest period of 10 days. The total acid consumed was 33 kg of concentrated sulfuric acid. 0.4% w/w solution. Copper extraction during this period was 9.85% (Fig. 3).

Copper extraction after inoculation of *T. ferrooxidans* MCM B-7 showed a steady increase with time up to 70 days, after which it declined. After a rest period of 10 days, copper extraction again increased up to the 126th day which was followed by a rest period of 13 days. The experiment was continued after the rest period and was terminated on the 180th day. A total of 19% copper extraction was obtained over the entire test period. This required a total addition of 51.79 kg concentrated sulfuric acid (as a 0.63% w/w solution).

Copper recovery by cementation was 90% of the total copper extracted i.e. 4.617 kg).

CONCLUSIONS

The results at the termination of the experiment show 19% copper recovered in 6 months time from 8 tons of ore. However, since copper extraction would continue beyond the 6-month period, these results indicate the copper leaching profile and *not* the absolute extraction obtainable from the ore. From these data, it was reasonable to project a total recovery of about 70% in a period of 6 years, if the leaching were carried out on a large scale so as to obtain 10 tons of copper per day. On the basis of these assumptions, the economics of the process for a large-scale heap leaching operation are given below.

Economics

For the purpose of this presentation, capital and operating costs for a 10 tons/day heap leaching operation are worked out. The size of the plant was kept to this level because it was believed that most new copper heap leaching operations would want to start off on a smaller scale until the process is well proven.

The plant specifications would be:

Capacity	10 tons/day
Quantity of ore	10,000,000 tons
Ore grade	Average 0.3% copper as chalcopyrite with some malachite and azurite.
Extraction	70%
Acid requirement	0.6% w/w
Extractable copper	21,000 tons
Year production	3,500 tons
Life of the leach plant	6 years
Operating days	350 days/yr
Power cost	Rs. 0.3/kwh (US $ 0.02)

The plant was designed to be constructed with a minimum capital cost and low operating cost as given below:

Capital costs

	Rs.	US $
Ore preparation	500,000,000	32,467,532
Solvent extraction-electrowinning plant	55,000,000	3,571,428
Buildings	1,500,000	97,402
Site preparation	10,000,000	649,350
Pumps and piping	10,000,000	649,350
Total	576,500,000	37,435,062

Operating costs

1) Labour:	No.
Labour 4/shift	12
Maintenance	2
Warehouse	2
Supervisor	2
Accounting/sales	2
Manager	1
	21

	Rs.	US $
Yearly cost for wages	504,000	2,467,532
2) Water & electricity/yr	3,000,000	194,805
3) Reagents	50,000,000	3,246,753
4) Overheads	1,500,000	97,402
Total operating cost per year	55,004,000	3,571,687

With a copper price of Rs 100,000/ton (US $ 6,493/ton) in India, the total earnings per year would be Rs. 350,000,000 (US $ 22,727,272). The net profit after deducting the operating costs would be Rs. 294,996,000 (US $ 19,155,584) per year.

If the capital of Rs. 576,500,000 (US $ 37,435,062) invested has to be repaid over 4 years at the flat interest rate of 15% then the Project must repay Rs. 922,400,000 (US $ 59,896,103). The profit of the Project over a period of 6 years would be Rs. 1,769,976,000 (US $ 114,933,506) at the current price of copper and hence, the net profit of the Project after repayment of the capital costs would be Rs. 847,576,000 (US $ 55,037,402).

It can be safely assumed that at present market rates prevailing in India, such an operation would be a success.

ACKNOWLEDGEMENT

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Table 1

Chemical and mineralogical analysis of Malanjkhand lean sulfide ore

Predominant minerals	Chemical analysis
Chalcopyrite	Total Copper = 0.37%
Malachite	Acid soluble copper = 0.03%
Azurite	Sulfidic copper = 0.34%
Pyrite	Total iron = 2.3%
Pyrrhotite	
Chalcocite	
Covellite	
Bornite	
Quartz	

Table 2

Laboratory-scale leaching experiments on Malanjkhand lean sulfide ore

Ore Sample	Culture used	Average copper extracted		
		Flask leaching	Air-lift percolator leaching	PVC column leaching
AI-53	*T. ferrooxidans* MCM B-7	36.7% in 60 days	13.42% in 120 days	14.32% in 180 days

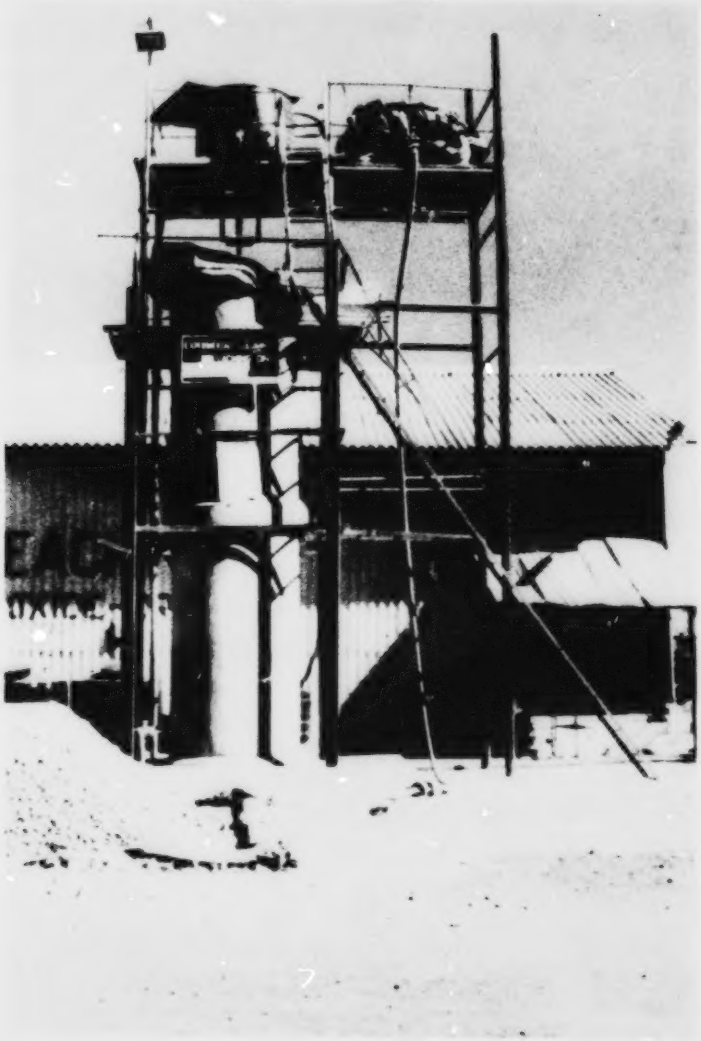

Fig. 1 Mine-site leaching of Malanjkhand Copper ore

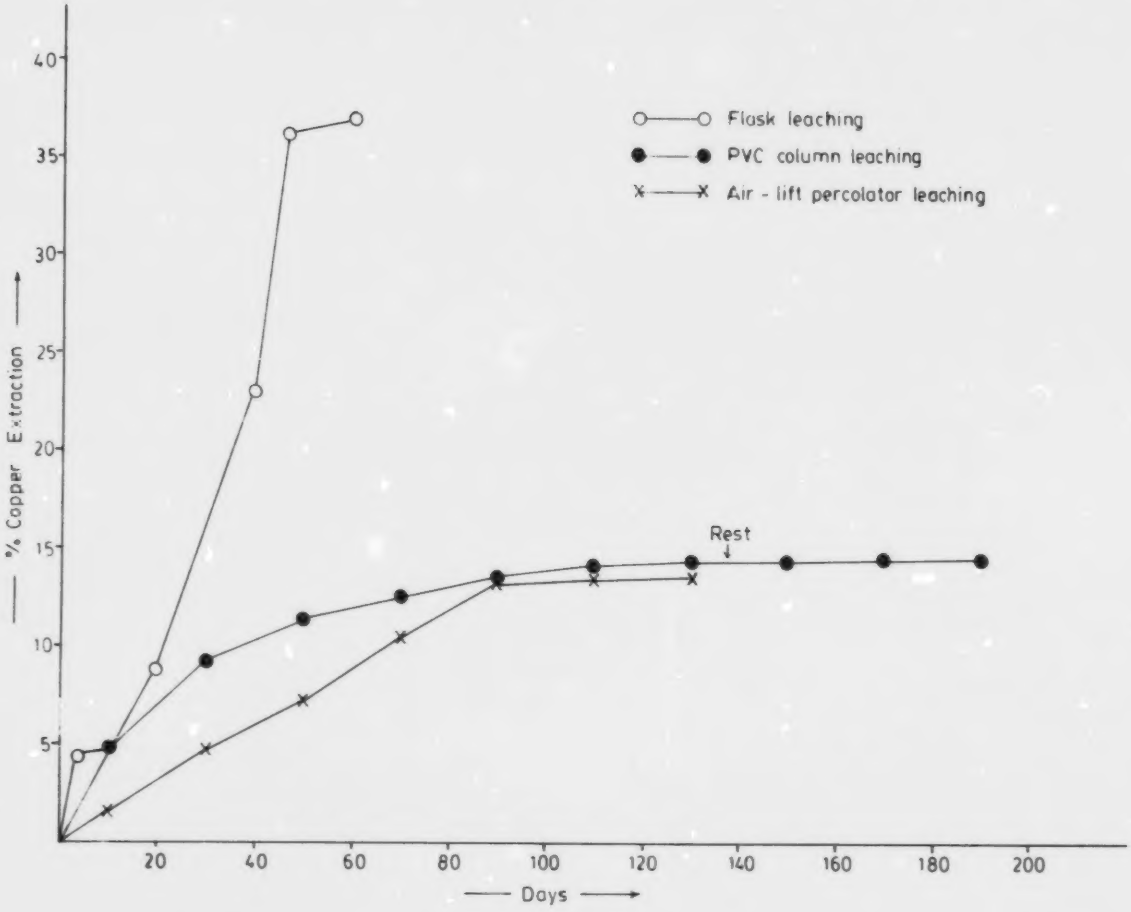

Fig. 2 Copper extraction in laboratory scale experiments

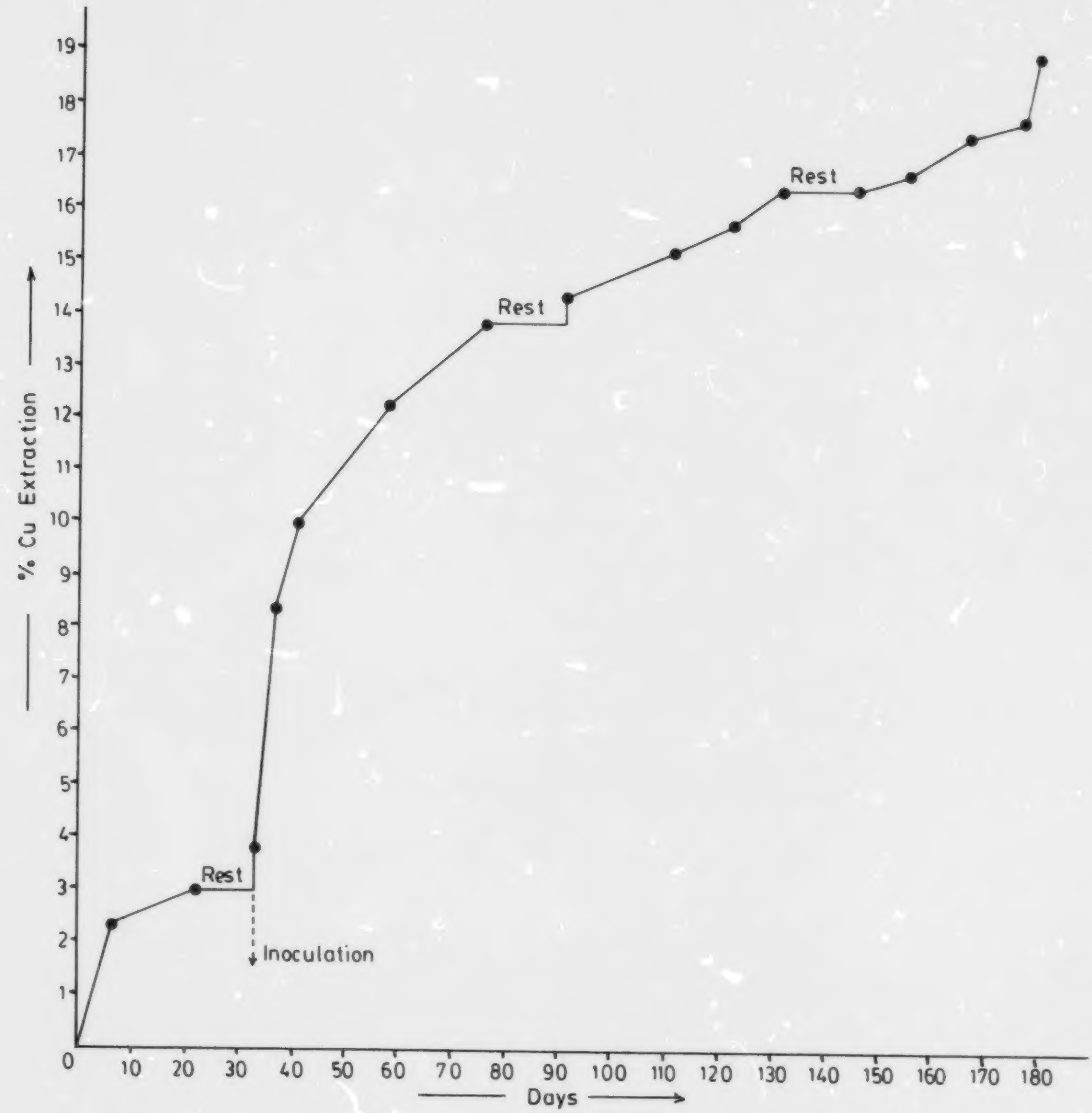

Fig. 3 Copper extraction in mine-site leaching experiment

WASTE TREATMENT

Biological Treatment of Mine and Mineral Processing Wastewater

By

Robert L. P. Kleinmann

U.S. Bureau of Mines, Pittsburgh Research Center, Pittsburgh, PA 15236

ABSTRACT

During the last two decades, the mining industry has greatly increased the amount it spends on pollution control. The Bureau of Mines estimates that the mining industry of the United States spends well over a million dollars a day on water pollution control alone. This is a trend that is likely to continue; some experts project that in the future, the industry will spend as much on pollution control as it does for the actual mineral extraction process.

LE TRAITEMENT BIOLOGIQUE DES EAUX DE MINE ET DES EAUX DE TRAITEMENT

Robert L.P. Kleinmann

Bureau des Mines, Centre de Recherche de Pittsburgh, Pittsburgh, PA 15236 E.-U.

RÉSUMÉ

Durant les deux dernières décennies, l'industrie minière a énormément augmenté le montant de ses dépenses allouées au contrôle de la pollution. Le Bureau des Mines estime que l'industrie minière américaine dépense beaucoup plus qu'un million de dollars par jour seulement pour le contrôle de la pollution de l'eau. Cette tendance va en toute probabilité se maintenir; quelques experts prevoient qu'à l'avenir l'industrie dépensera autant d'argent pour le contrôle de la pollution que pour l'extraction minérale.

Introduction

The application of biotechnology to water pollution control is a way to reduce this economic burden. Biological water treatment with microorganisms, or a mix of microorganisms and higher plants, is already being utilized at hundreds of mine sites to reduce chemical treatment requirements. In addition, private industry is already beginning to market various forms of immobilized biomass for removal of metals from wastewater streams, even as research continues in this area.

The mechanisms of treatment, in both the living and immobilized systems, consists of ion exchange, chelation, intracellular uptake and storage, metal precipitation on cell surfaces, metabolism-dependent oxidation or reduction, pH adjustment and indirect removal mechanisms associated with excreted substances, such as polysaccharides, organic acids and hydrogen sulfide. Typically, many of these processes are going on at the same time; the relative importance of each changes with time, water quality and environmental factors (e.g. temperature). Much of the research to-date has documented changes in water quality, without a detailed study of the process; as a result, it is difficult to scale up, predict long-term performance or optimize wastewater treatment. This situation is changing, however, as research proceeds.

Use of Constructed Wetlands

In general, biological treatment utilizes naturally-occurring organisms, thereby avoiding the technical, legal and economic drawbacks of attempting to tailor species to the given wastewater. Nutrients can be provided, if necessary, but the systems are typically designed to be inexpensive and to require very little maintenance.

Biological treatment of mine wastewater is typically conducted in a series of small excavated ponds that resemble, in a superficial way, a small marsh area. Over 400 of these wetland water treatment systems have been built on mined lands. Early constructed wetlands were planted with *Sphagnum*, in an attempt to simulate natural bog-type wetlands. Experience gained in the field has led to the more prevalent use of emergent plants, especially cattails (*Typha*), and greater consideration of the biochemical processes that occur in the substrate materials.

Accumulation of metals by growing plants varies considerably depending on the metal and the plant in question. *Sphagnum* has a tremendous ability to accumulate iron, but if exposed to even moderate concentrations of iron, it absorbs so much metal that it petrifies (Spratt and Weider, 1988). *Typha* is much more tolerant of mine water precisely because it is not as efficient in accumulating metals. Sencindiver and Bhumbla (1988) calculated that the bioaccumulation of 19,506 g/g of iron in the *Typha* rhizomes at a site receiving 10 mg/L Fe accounted for only 0.2 pct of total annual inflow of iron. Bioaccumulation of manganese by *Typha* was even less significant.

Algae also accumulate iron and manganese. When calculated on a dry weight basis, the resultant accumulated concentrations appear to be quite impressive. Kepler (1986) measured concentrations as high as 56,000 mg of manganese per kg (dry weight) of algae in samples of *Oscillatoria*. We have collected numerous *Microspora* samples that contain, on a dry weight basis, 30,000 - 90,000 mg/kg of manganese. However, algae biomass in most wetlands is very limited, so that its contribution to metal removal will rarely reach significant levels. Calculations show that very productive algal systems accumulating manganese at 50,000 mg/kg would still only remove 4 mg/L of manganese from AMD, assuming currently-sized wetland systems (Hedin, 1989).

Plants and organic substrate do, however, provide sites for bacterial attachment and colony development. In addition, the large surface area to volume ratio of most wetlands enhances aeration of surface waters, which is often a prerequisite for the bacterial oxidation of metals. Emergent plants are also capable of aerating the sediments by releasing oxygen from their roots. *Typha* roots are often coated with iron oxyhydroxides, which some researchers believe is due to plant-induced oxygenation of the rhizosphere and the activity of iron- oxidizing bacteria (Taylor, Crowder and Rodden, 1984; Sencindiver and Bhumbla, 1988). Finally, the organic detrital material and the carbon excreted by plants provide food for heterotrophic metal-oxidizing bacteria in the aerobic zone and for sulfate-reducing bacteria in the anaerobic bottom waters.

At most sites where biological water treatment is practiced, the principal contaminant being removed is iron. Thiobacilli and other aerobic iron-oxidizing microbes, are very effective in removing iron. However, the removal of most of the iron eliminates much of the need for chemical treatment, but does not, by itself, eliminate its necessity. Indeed, only about 20 pct of the constructed wetland systems discharge water that completely meets the U.S. effluent limits of no net acidity, pH 6-9, average total iron ≤ 3 mg/L and average total manganese ≤ 2 mg/L. At most sites, supplemental chemical treatment is necessary, but the chemical treatment costs are much lower than they would be if biological treatment had not been utilized. In fact, most operators find that the costs of constructing the wetlands are recovered within one year through savings in chemical usage.

In most of the wetlands constructed to treat coal mine drainage, flow is predominantly above the substrate, so that only a small fraction of the water passes through the anaerobic zone. The water in the substrate is markedly different from the overlying surface water a few centimeters higher. For example, in the pore waters, the pH is 3-5 units higher and dissolved iron concentrations can be 50-99 pct lower. The Bureau of Mines is currently experimenting with wetland designs that utilize subsurface flow to expose a higher proportion of the water to be treated to the anaerobic zone. Preliminary winter season results from a site with water of pH less than 3, acidity values of 1,000 mg/L (as $CaCO_3$), and iron concentrations of 150-200 mg/L, indicate that promoting flow through the anaerobic zone raised the pH of the water to near-neutral, decreased acidity to near zero and decreased iron concentrations to 10-20 mg/L. Ongoing work will determine how much of this improvement is due to relatively short-termed ion exchange effects and how much is due to sulfate-reduction, but the results to date are in sharp contrast to the effectiveness of conventional flow above the substrate.

POTENTIAL APPLICABILITY TO METAL MINE DRAINAGE

When extending the results from coal mines in the eastern United States to the possible treatment of metal mine effluent waters, one consideration is the effect of high metal concentrations on wetland performance. Regarding tolerance, the literature is encouraging. *Typha latifolia* can tolerate copper concentrations up to 50 mg/L and nickel concentrations greater than 150 mg/L without difficulty (Taylor and Crowder, 1983a). *T. latifolia* shoots grown in a solution of 100 mg/L Cu (as an EDTA complex) accumulated 127 ± 28 *µg/g* Cu in their leaves and 2,364 ± 209 *µg/g* Cu in their roots, but displayed reduced leaf growth when the copper concentrations in the leaves reached 80 *µg/g* (Taylor and Crowder, 1983b; Taylor and Crowder, 1984). *Typha* has been reported growing in a mine water marsh where the sediment contained 3,738 *µg/g* Cu and 9,372 *µg/g* Ni (Taylor and Crowder, 1983b). Kalin and Van Everdingen (1987) have successfully demonstrated that *Typha* can be transplanted into acid water with a pH range of 2.9 to 5.7, a mean zinc concentration of 221 mg/L and a mean copper concentration of 21 mg/L. However, hydroponic transplantation has been unsuccessful (Kalin, 1988). Other wetland plants may be somewhat less metal-tolerant. For example, cadmium retarded the growth of *Sphagnum fimbriatum* at 0.1 mM (11 mg/L) but not at 0.01 mM. Lead at 1 mM (207 mg/L) strongly retarded the growth of *Sphagnum fimbriatum* but was tolerated at this concentration by *Fetusca ovina and Hordeum vulgaris* (Gignac and Beckett, 1986; Lee, Jonasson and Goodfellow, 1984). Certain algal species can also tolerate significant concentrations of lead, copper and zinc (Gale and Wixson, 1979; Kalin, 1988).

Bioaccumulation of metals, though potentially significant in slightly contaminated waters, is biomass-limited. Metal uptake by wetland plants has been reviewed by Chan and others (1982); cited plants include pickleweed (*Salicornia pacifica*), which can remove 0.016 to 0.024 kg Cd/ha, reed canary grass (*Phalaris arundinacea*), which can remove 0.69 kg Cu/ha and a sedge (*Carex stricta*) which can remove 0.67 kg Ni/ha. For the more toxic metals, bioaccumulation may actually cause problems, for it could result in the chronic poisoning of foraging animals.

Metal removal by sulfate-reduction appears to be much more promising. Although sulfate-reducing bacteria are inhibited at low pH, their activity increases the pH in their immediate environment, allowing their activity in the sediments beneath extremely acidic waters (Herlihy and others, 1987; Satake, 1977). Recent work in our laboratory indicates that sulfate-reducing bacteria are also relatively tolerant of high heavy metal concentrations. Preliminary screening tests indicate that the sulfate-reduction potential is not inhibited by nickel, lead or zinc concentrations as high as 200 mg/L. Cadmium, however, appears to be toxic at concentrations greater than 35 mg/L. Important information about the sulfate-reducing bacteria must still be determined, such as the rate of hydrogen sulfide generation, sensitivity to parameters such as temperature and water chemistry fluctuations, and the nature of organic compounds required for their activity. The latter is critical because partial breakdown of the organics by fermentative bacteria will probably be necessary; their tolerance and sensitivity may therefore be important as well.

We have begun laboratory experiments in which anaerobic columns filled with organic compost and a mixed culture of anaerobic bacteria are being exposed to various concentrations of dissolved metals in simulated AMD. Similar experiments with iron and sulfate-enriched water revealed that iron removal was strongly associated with losses of dissolved sulfate; stoichiometry indicates this was due to sulfate-reduction and the subsequent formation of iron sulfides.

It should be recognized that precipitation of sulfide minerals is only going to continue if there is sufficient organic matter to maintain an anaerobic environment. If seasonal decay of emergent species and the excretion of organic compounds by plants is inadequate, then periodic addition of selected organic materials may be necessary. The nature of this requirement will be tested in the near future. If, however, one can afford to periodically add organics, there are potential advantages to biological treatment without wetlands, especially for metal mine drainage. First, the areal requirements can be reduced, for instead of a shallow system, one can emphasize depth. For example, an abandoned pit could serve as the primary biological treatment basin. Second, seasonal variations in performance could be minimized. It may be possible to treat mine water in a pipeline-type reaction vessel filled with composted organic waste; presumably, this could even be done inside of an active or abandoned section of an underground mine. In regions with long, harsh winters or limited land surface, or if bioaccumulation becomes a concern, both approaches would be preferable to a constructed wetland approach. During the next few years, in addition to our wetland studies, we will be evaluating the feasibility of treating metal mine drainage biologically without wetlands.

Conclusion

Biotechnology offers tremendous potential for the mineral industry. Within the next ten or fifteen years, new processes will be developed and put into practice that are based on the laboratory work of today. However, in the pollution control area, the revolution has already begun. Just as municipal wastes and many industrial wastes are treated biologically, so too can we anticipate that, more and more, mine wastewater will be treated biologically.

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CONSTRUCTED WETLANDS IN PENNSYLVANIA AN OVERVIEW

by

William W. Hellier, Jr.
Department of Environmental Resources
Commonwealth of Pennsylvania

ABSTRACT

Some 144 wetlands have been constructed in an attempt to abate pollution from mine sites in Pennsylvania. Data from 48 of the constructed wetlands were compiled and analyzed. Wetlands that have average influent net acidities of less than 200 mg/L $CaCO_3$ are effective in meeting effluent Fe limits of 7 mg/L and in reducing Mn to below 20 mg/L, as compared to effluent limits of 4 mg/L. Wetlands were not generally observed to have effluents that conformed to pH or alkalinity requirements in cases where the influent did not already conform. A minimum design surface-area-to-flow-rate ratio of 20 $m^2/(m^3/day)$ appears necessary. In terms of percent removal of metals, similar effects can be seen, with efficiencies being less predictable below the threshold values. Further empirical data are necessary to confirm these criteria. Based on the available data, several sites examined have shown progressive improvement in performance. Final decision on wetlands effectiveness rests on the analysis of more and better data.

BASSINS DE DÉCHARGE ARTIFICIELS EN PENNSYLVANIE – SURVOL

W. W. Hellier, Jr.

Département de Ressources Environnementale,
Commonwealth of Pennsylvanie, É.-U.

RÉSUMÉ

Quelques 144 bassins artificiels ont été construits en vue de réduire la pollution causée par les sites miniers de la Pennsylvanie. Les données relatives à 48 de ces bassins ont été compilées et analysées. Les bassins dont l'affluent présente des moyennes d'acidité nettes inférieures à 200 mg/L de $CaCO_3$ répondent efficacement aux limites de Fe de 7 mg/L dans l'effluent tout en réduisant la concentration de Mn à moins de 20 mg/L, comparativement aux limites de 4 mg/L prescrites pour l'effluent. Les exigences de pH et d'alcalinité des effluents des bassins ne semblaient pas, en général, être conformes dans les cas où l'affluent n'était pas lui-même conforme aux exigences. Il semble nécessaire d'avoir un rapport de la surface nominale minimale en fonction du débit qui correspond à 20 $m^2/(m^3/jour)$. En termes de pourcentage de métaux extraits, on peut observer des effets similaires, les rendements étant moins prévisibles lorsque les valeurs sont inférieures à celles du seuil. Il est nécessaire de disposer de données complémentaires pour confirmer ces critères. En fonction des données obtenues, plusieurs sites étudiés ont présenté des améliorations de rendement. La décision finale quant à l'efficacité des bassins artificiels dépend des résultats d'analyse d'un plus grand nombre de données et plus complètes.

INTRODUCTION

Postmining discharges present a major environmental problem in Pennsylvania. Current regulation requires that all postmining discharges conform to effluent limitations of $6.0 \le pH \le 9.0$; $Fe \le 7.0$ mg/l; and, for mining which occurred after 08/03/77, $Mn \le 4.0$ mg/l. Thus far, the Department has documented approximately 756 postmining discharges not conforming to these limits for which a current Permittee has continuing liability.

Based on observations of workers such as Tarleton *et al.* (1984) and Kleinmann (1985), wetlands appeared to present possible alternatives to conventional chemical treatment. Accordingly, beginning in late 1983 and early 1984, several Permittees with pollutant discharges constructed artificial wetlands to treat the discharges. Some 144 wetland systems of various sizes and configurations presently exist.

Because the wetlands systems evolved as efforts to reduce treatment costs and were generally followed by conventional treatment systems, only a minority of the systems have plans and specifications on file. Furthermore, a formal monitoring plan was established only in a few cases. While selected systems from the Pennsylvania inventory have been the subject of study by the U.S. Bureau of Mines and several members of the academic community , the data for all of the systems have remained in the Department's files; and only within the past year has a systematic compilation and analysis been attempted. The Department is presently assembling the existing data and will determine what additional data are needed to help advance the science of wetland treatment.

This presentation will give an overview of 48 of Pennsylvania's constructed wetlands, will present some preliminary findings, will consider some case studies, and will suggest some added information to be gathered.

Beginning in November of 1986, the Department has required the submittal of flow data for monitoring points on proposed permit applications. Historically, where flows have been reported, they have been based on the entire range of techniques from accurate flume or weir data to visual estimates. Monitoring is required by the permits for approved monitoring points and final treated discharges, and not for raw discharges into treatment systems or between treatment ponds.

As a consequence, the quantity of data varies from file to file. Conclusions are drawn based on analysis of the data as found, realizing that for the 48 cases considered, there was an average of 19 separate influent samples taken during the operation of the wetland, ranging from a low of 2 to a high of 68. The corresponding average for effluent samples was 22, with a low of 1 and a high of 165. In 17 cases, there were fewer than 10 influent samples; in 12 cases, between 10 and 19; and in 19 cases, 20 or more. The corresponding figures for effluent samples were 16, 12, and 20. The data for each particular case are available for examination.The trends discussed herein do not appear to be heavily dependent on the quantity of data, but it is realized that caution must be exercised in any interpretation based on sparse data. The Department will obtain the added data necessary to make the averages more clearly representative.

In some cases, the wetland influent is a discharge or seep over which one or more wetland sections, or cells, has been constructed, making flow and quality determinations difficult to obtain. In only a few cases have groundwater or rainfall contributions been accurately determined. In some cases, surface runoff has not been excluded.

Sufficient flow data for flow weighting of the quality parameters was available for only 12 of the influents and 14 of the effluents for the 48 that were studied. In the remaining cases, flow considerations had to be based on only a few measurements or estimates.

The chemical parameters were obtained from samples collected and analyzed by the Department and by coal companies. The Department's samples were analyzed using the methods in *Standard Methods for the Examination of Water and Wastewater* (1985).

RESULTS

Overall Observations on the 48 Selected Sites:

The average flow and quality parameters for the influent and effluent of 48 selected wetland systems are given in Table 1. In cases where sufficient flow data were available, the average concentrations are flow-weighted; in those cases where flow data are not available, arithmetic averages were used. A "yes" in the Table indicates that flow weighting was used, and "no" indicates that it was not.

Figures 1 through 3 depict the performance of the wetlands based on the averages of the data for each of the selected wetlands, and Figures 4 through 6 depict the performance of three selected wetland systems. Because the compliance of the discharges with effluent limits is the important issue from a regulatory standpoint, and because surface settling rates and net alkalinities were thought to play an important role, effluent concentrations of Fe and Mn were plotted versus the surface settling rate, or wetland area divided by influent flow rate, A/Q, and versus net influent alkalinity (Fig. 1). The average effluent Fe and Mn concentrations declined as the net alkalinity increased. Once the net alkalinity exceeded -200 mg/l (i.e. net acidity was less than 200 mg/l), in only two cases did the average effluent Fe concentration fail to meet effluent limits. The same effect is seen for manganese, except that the threshold value of around 20 mg/l is higher than effluent limits by a factor of 5. For net alkalinities less than -200 mg/l, performance was much less consistent and efficient.

The effluent Fe and Mn concentrations were substantially lower when A/Q was greater than 10 to 20 $m^2/(m^3/day)$ than when A/Q was less. In all but 2 cases where the A/Q value exceeded 20, and in most cases where it exceeded 10, the average effluent Fe concentration met effluent limits. The concentration of Mn, while relatively low, was not generally found to meet effluent limits.

The data from Fig. 1 suggest a minimum design criterion of 10 to 20 $m^2/(m^3/day)$ as an engineering criterion for sizing of constructed wetlands. The higher figure compares with past suggestions of 3.4 $m^2/(m^3/day)$. Intuitively, the sizing of any treatment system ought to depend of the amount of pollutant to be removed. Therefore, plots were generated of the percentage removal rates of Fe and Mn as functions of net alkalinity, pH, and surface settling rate (A/Q). (Figs. 2 and 3).

Percent removal for Fe and Mn removed per day generally exceeded 50%. While no correlation between percent Fe or Mn removal and A/Q was evident, when $A/Q \geq 20m^2/(m^3/day)$, metals removal always ≥ 75% (Fig. 2). While pH and alkalinity increases enhanced Fe and Mn removal (Fig. 3), this might simply reflect chemical precipitation that would occur whether or not a wetland had been constructed. Attempts to further quantify the relation between percent removal or absolute amount of metal removed and the surface settling rate were not successful, because of the scatter in data. Compilation of data under controlled conditions is recommended in order to better define a possible design criterion. In cases where the influent pH was less than 4, the wetland effluent pH did not conform to effluent criteria. In several cases where the influent pH was between 4 and 6, the effluent pH conformed,while in no case where the influent pH was greater than 6 did the effluent pH fail to conform. The data indicate that for highly acidic conditions, wetlands systems cannot be relied upon to meet effluent criteria relative to alkalinity.

Case Studies:

Case studies have shown progressive improvement in effluent metals concentrations in constructed wetlands. The wetland case study in Figure 4 is achieving effluent Fe that meets effluent limits, and Mn has steadily declined to within effluent limits as well. The sulfates have remained virtually unchanged through the wetland, while there has been some pH improvement.

Two postmining discharges are combined above the wetland. The substrate was rotted, weathered hay, without the use of any limestone,or mushroom compost, according to Barrett (1989). *Juncus* and *Equisetum* exist, with *Typha latifolia* beginning to establish themselves. The *Sphagnum* that was originally planted did not become well established.

There are nine wetland cells in series at progressively lower elevations. There was no provision for diversion of storm water around these wetlands. However, itis evident from a comparison of sulfates that the influent and effluent average flows are approximately equal. The flow of the combined discharges is rather copious during rain events. Iron oxide precipitate persists in the influent ditch and through the fifth cell; it becomes less in the sixth cell, and is absent in the seventh through ninth cells. It appears from the manganese removal that biological processes can account for abatement.

Design criteria can be developed to establish reserve retention areas or maintenance schedules to account for the expected accumulation of solids due to Fe and Mn removal. For example. in this case study, since Fe is being removed at an average of 385 gm/day ,and Mn at a rate of 143, solids could be washed from the system during periods of copious precipitation, or they could accumulate in the wetland at an estimated average rate of about $2x10^{-4}$ m^3/day. If a 1.0 cm accumulation is tolerable, the solids would have to be removed or the system would have to be otherwise maintained every 10 years or so.

A second case study system consists of a settling ditch followed by three ponds with wetland species on their peripheries. The backfilled area has been enhanced by addition of alkaline material at the lowwall barrier. The metals removal is progressive (Fig. 5), and is very noticeable with respect to the difference between Fe concentrations in the influent to cell 3 and the final effluent. Effluent Mn concentrations are trending downward, and the final cell (cell 3) supports fish life.

The system in the third case study appears to exhibit seasonal behavior,as shown in the Fe and Mn plots in Fig. 6, and as pointed out by Fisher (1989); seasonal effects have been considered by McHerron (1985), and are under study by Hedin (1989). This system has had few if any man-made improvements during the period of data compilation.

CONCLUSIONS

1. In 38 of the 48 systems considered, the average effluent Fe concentration conformed to effluent limits. This suggests that constructed wetlands can be productively utilized as systems to reduce iron as a pollutant from mining discharges.

2. Based on the data, a minimum size of between 10 and 20 $m^2/(m^3/day)$is suggested for constructed wetlands. This is higher than the past guideline of 3.4 $m^2/(m^3/day)$ by a factor of between 3 and 6. This reflects concentration effects, as well as corrections for "effective" area.

3. Rather than designing wetlands on the basis of size alone, it was thought that design should be based on a dependence of percent removal of pollutants such as Fe or Mn on surface settling rates, A/Q, in a manner analogous to sewage treatment. Attempts to quantify a possible dependence for Fe or Mn removal were unsuccessful, based on the limited data available,although removal efficiencies were generally seen to be less predictable with A/Q below 10 to 20 $m^2/(m^3/day)$, in agreement with the second conclusion. Further empirical data both from existing systems and from systems built to study removal rates under controlled conditions should provide quantitative design criteria.

4. Based on the data, it appears that adjustment of influent alkalinity to not less than -200 mg/l $CaCO_3$ might improve the effectiveness of wetlands treatment for Fe removal. This could be accom-

plished by means of incorporation of reject lime or other alkaline material into the backfill, or by a pretreatment system. Further study of this concept would be desirable.

5. Conclusions drawn thus far must be tempered by their dependence on the limited data available. It is recommended that the data be augmented for all existing wetlands systems by requiring that accurate flow data be collected with all quality data for the influent and effluent at a minimum. For many of the systems studied, the quality data are sparse and therefore may not be representative. It is recommended that quality and flow data be gathered at least monthly for at least 24 consecutive time periods, so as to encompass two complete water years. This information is especially critical in refining the design criteria for sizing.

6. Periodic maintenance of the wetlands systems may be needed from time to time. As more data become available, design criteria for maintenance schedules and added solids retention volumes can be developed.

7. Further research should be encouraged on new or existing systems. For example, as shown by Stumm and Morgan (1981), as well as Morgan *et al.* (1985), the Mn removal reaction is autocatalytic, so that seeding of a wetland with MnO_2 could be considered. In cases such as Case Study 2, this might prove less deleterious to existing fish life than use of conventional treatment to achieve effluent manganese limits. The implantation of manganese removing microorganisms is also being considered by Vail (1989) at one of the Pennsylvania sites.

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Thanks are due to Bureau Director Ernest F. Giovannitti for his continued support throughout this study. Karl K. Sheaffer provided valuable guidance as well.Thanks also to Keith Brady for his valuable critique and comments, and to John Hulsberg and Michael J. Klimkos for their help with computations. The entire field staff of the Bureau of Mining and Reclamation has been most cooperative. Especially helpful have been Inspectors Walter A. Kuzemchock and James McDonald. Thanks are also due to summer intern Kelly L. Fagley for data compilation. Several private consultants have given generously of their time, including Wilson Fisher, Jr., Terry A. Rightnour, Dean W. Barrett, and Jennifer Shufran. The various coal operators, such as McDonald Land and Mining Co, Inc., and Bradford Coal Co., Inc., have been most helpful. The author is indebted to the staff at the U.S. Bureau of Mines, particularly Dr. Robert Hedin, David Hyman, Jay W. Hawkins, and Pamela McIntire for their review and critique.

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TABLE 1

AVERAGE PERFORMANCE DATA FOR 48 WETLANDS SYSTEMS

Areas in m^2, flow rates, Q in m^3/day, pH dimensionless, other parameters in mg/l; alkalinity (alk) as $CaCO_3$.

		INFLUENT								EFFLUENT							
Wetland System	Wetland Area	No. of Samp	Q	pH	Net Alk	Fe	Mn	SO4	Wtd?	No. of Samp	Q	pH	Net Alk	Fe	Mn	SO4	Wtd?
1	1031	4	273	6.2	73	11.8	3.4	143	no	6	50	6.2	65	7.9	4.0	110	no
2	70820	8	273	6.6	98	9.6	9.0	605	no	4	545	6.7	57	0.7	3.9	448	no
3	1301	4	27	4.3	-167	1.0	43.6	884	no	5	55	5.6	-34	2.2	28.4	920	no
4	609	10	98	5.8	-37	23.9	27.8	743	no	9	98	6.3	16	1.8	21.7	638	no
5	1394	2	5	5.9	-33	21.3	10.1	392	no	1	11	6.3	20	1.3	0.5	45	no
6	91	2	16	6.5	138	3.8	8.0	204	no	4	71	6.8	72	0.5	3.5	198	no
7	74	11	13	5.1	-156	0.3	21.8	1479	no	4	0	6.1	46	0.3	10.0	1578	no
8	1394	15	16	6.4	62	1.3	8.9	473	yes	16	3	6.6	49	0.8	6.7	525	yes
9	2787	43	158	3.4	-1450	32.0	102.3	3664	no	165	158	3.3	-748	14.9	90.9	3217	yes
10	822	37	234	6.3	169	29.5	24.1	945	yes	28	524	6.4	159	3.6	19.5	820	yes
11	1892	11	65	5.3	-40	0.3	6.6	502	yes	18	65	6.1	16	0.5	6.6	285	yes
12	920	11	65	5.3	-40	0.3	6.6	502	yes	16	168	6.9	82	0.1	1.7	307	yes
13	987	31	62	6.1	-5	23.4	23.9	612	no	28	78	6.7	202	2.7	18.7	417	no
14	190	14	27	3.6	-1041	47.8	97.1	2130	no	7	22	3.7	-253	10.6	95.2	2113	no
15	557	9	74	6.4	72	7.4	14.7	275	no	25	74	7.3	133	2.6	15.2	411	no
16	369	17	33	3.1	-400	20.7	7.2	367	yes	24	33	3.6	-78	7.8	7.0	465	yes
17	318	18	27	4.8	-97	0.3	12.0	906	yes	17	44	6.0	61	0.5	8.6	917	yes
18	838	41	49	5.3	-2	0.8	7.0	1021	yes	25	82	5.6	84	0.4	4.7	964	yes
19	753	5	5	5.3	-5	0.3	13.2	743	no	9	5	6.0	222	6.0	5.5	663	no
20	232	7	22	6.3	121	13.1	4.2	181	yes	7	22	7.1	101	0.5	0.8	190	yes
21	2993	17	109	6.3	113	15.8	8.7	230	no	17	109	6.6	134	3.9	9.0	393	yes
22	2861	10	136	6.5	94	9.6	3.9	227	no	11	136	6.7	71	2.6	1.5	120	no
23	1858	5	44	7.4	2434	1.3	15.1	1566	yes	18	50	5.7	345	5.2	15.3	1495	no
24	167	3	11	4.2	-414	0.3	75.5	1272	no	26	11	5.4	-23	0.8	41.2	1519	no
25	836	3	109	3.3	-722	37.9	88.6	1208	no	6	109	3.6	-311	32.2	82.5	1054	no
26	186	15	55	3.8	-243	17.4	57.6	1173	no	8	55	5.0	-35	0.8	19.6	502	no
27	929	19	8	3.0	-2018	270.6	43.9	2239	yes	18	18	4.4	-67	6.7	6.5	1722	yes
28	1877	55	191	2.9	-2144	232.3	40.0	?	no	55	191	3.4	-253	18.2	38.6	?	no
29	1877	37	191	3.3	-1014	32.1	65.7	?	no	37	191	4.4	-131	3.1	50.3	?	no
30	260	34	191	4.4	-672	19.3	37.3	?	no	34	191	5.5	-33	0.6	13.6	?	no
31	325	39	191	3.1	-844	44.5	58.8	?	no	39	191	3.7	-238	4.3	62.2	?	no
32	530	68	160	6.0	80	7.8	6.2	412	no	67	160	6.1	73	0.8	3.6	403	no
33	297	20	44	3.1	-750	95.7	76.4	1380	no	24	44	3.2	-271	42.4	69.7	1291	no
34	111	20	153	3.6	-480	78.0	38.6	890	no	17	153	3.5	-136	32.2	36.8	873	no
35	576	20	60	2.8	-1094	68.9	82.0	1346	no	22	60	3.3	-228	23.2	63.1	1306	no
36	4645	5	1	6.1	71	3.4	29.7	889	no	4	1	6.5	26	1.0	8.5	729	no
37	279	5	27	5.2	-37	38.1	6.5	390	no	7	14	4.2	-25	5.0	3.8	137	no
38	3902	24	44	6.8	459	21.1	6.1	749	yes	24	294	7.2	251	1.4	7.3	996	yes
39	1331	7	8	6.3	122	11.3	3.6	119	no	12	8	6.5	104	4.8	3.7	122	no
40	753	6	1	6.2	49	1.5	18.7	158	no	5	1	7.8	130	0.2	26.6	737	no
41	697	16	55	3.3	-594	12.5	40.5	1016	no	19	55	3.6	-129	4.4	36.3	1072	no
42	209	4	44	5.9	16	6.0	22.0	380	yes	4	60	6.5	71	0.7	0.6	236	yes
43	3605	22	100	6.4	101	14.4	14.6	232	no	38	100	6.6	77	1.6	8.0	200	no
44	4877	24	47	3.1	-1470	249.9	49.4	1435	no	27	72	3.4	-187	36.3	20.9	634	no
45	418	39	55	4.1	1	34.4	13.7	916	yes	24	120	4.3	0.4	35.4	12.8	930	yes
46	2007	51	376	5.9	134	10.1	14.7	516	no	54	55	6.1	37	6.8	2.2	509	yes
47	279	8	27	5.6	31	3.9	10.9	459	no	10	27	6.4	101	1.0	8.0	430	no
48	1579	28	12	3.0	-1035	87.5	99.0	3248	no	28	23	3.8	-205	39.2	132.0	3044	no

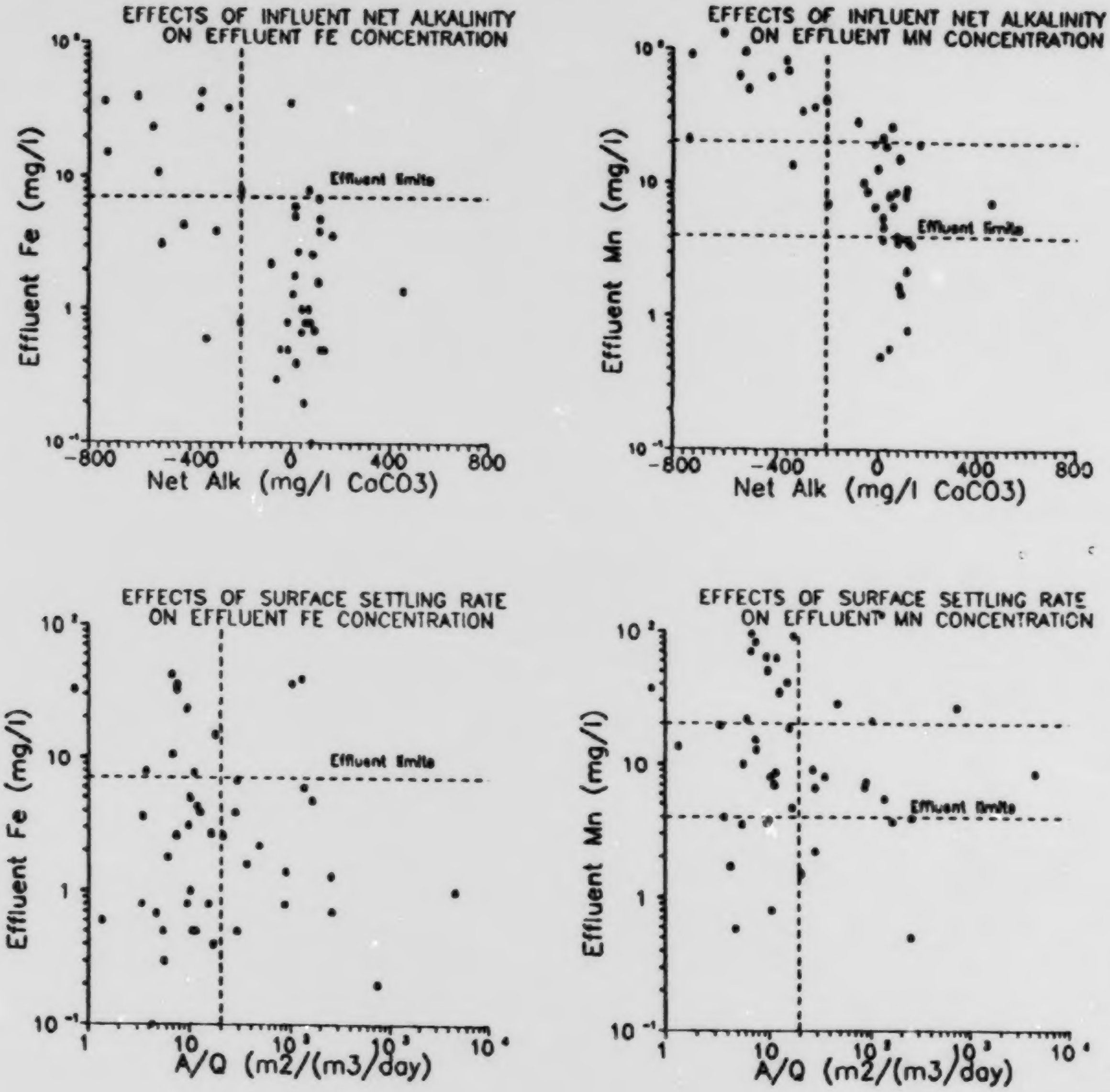

Fig. 1 Effects of Influent Net Alkalinity and Surface Settling Rate (Area/Flow) on Effluent Fe and Mn Concentrations.

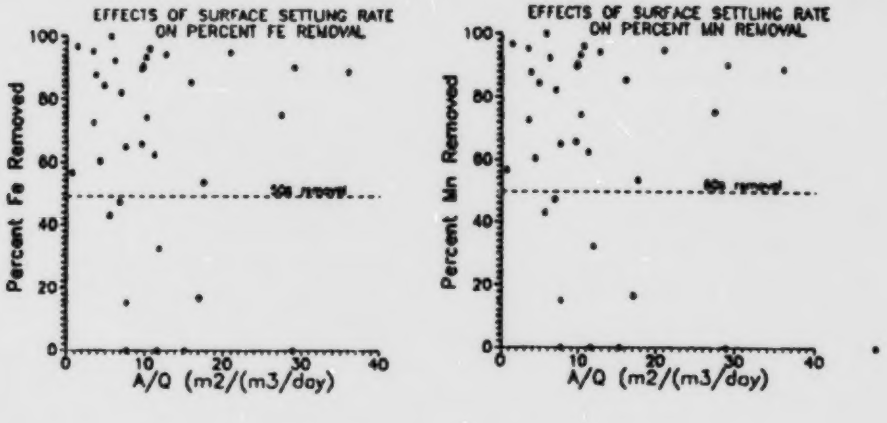

Fig. 2 Effects of pH, Influent Net Alkalinity, Flow, and Influent Fe concentration on the Quantity of Fe removed. The dividing line is for reference, and shows 50% removal.

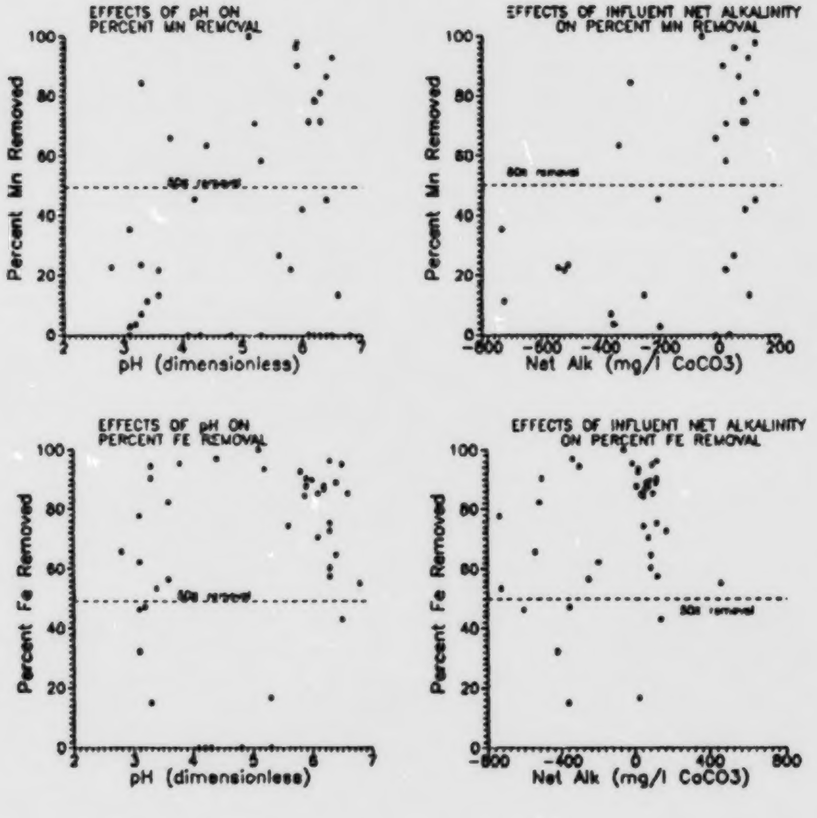

Fig. 3 Effects of pH, Influent Net Alkalinity, Flow, and Influent Mn concentration on the Quantity of Mn removed. The dividing line is for reference, and shows 50% removal.

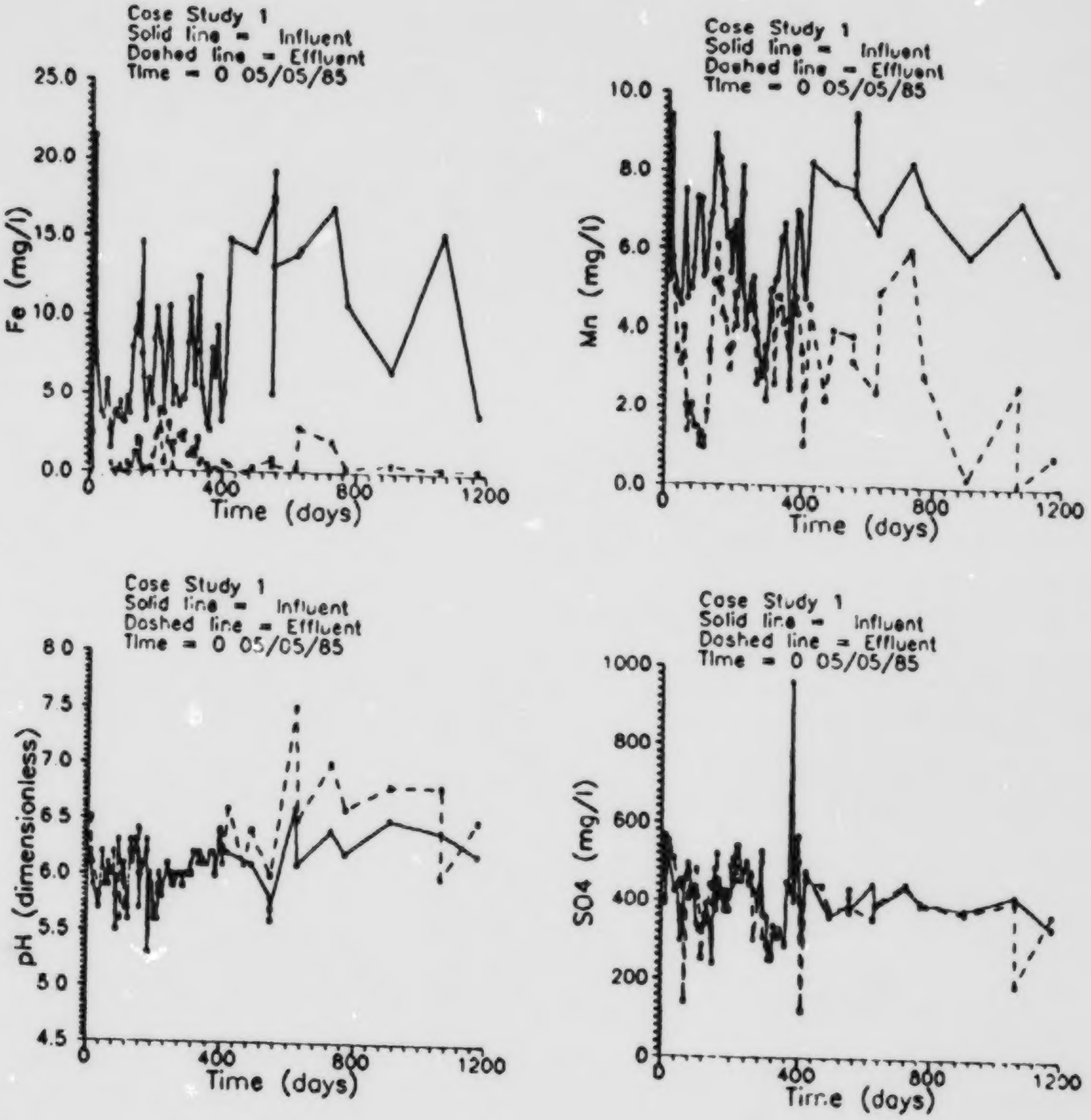

Fig. 4 The performance of a wetland as a function of time: Case Study 1. Note especially the current conformity of Mn to effluent limits. The consistency of $SO^{4=}$ concentrations suggests that little dilution is occurring.

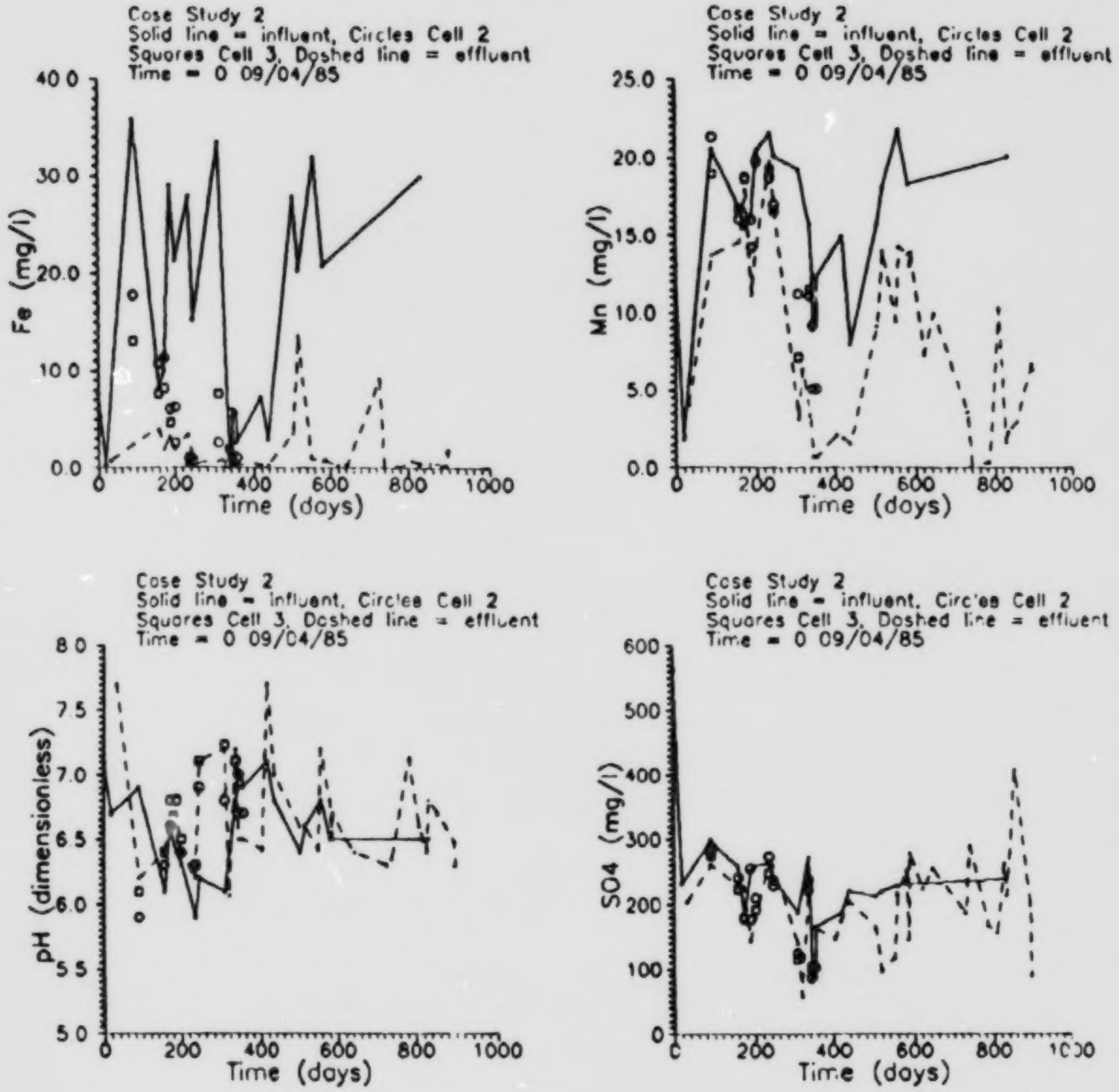

Fig. 5 The performance of a wetland as a function of time: Case Study 2. Performance is progressive, but Mn does not meet effluent limits.The extreme downstream wetland cell of three in series supports fish life.

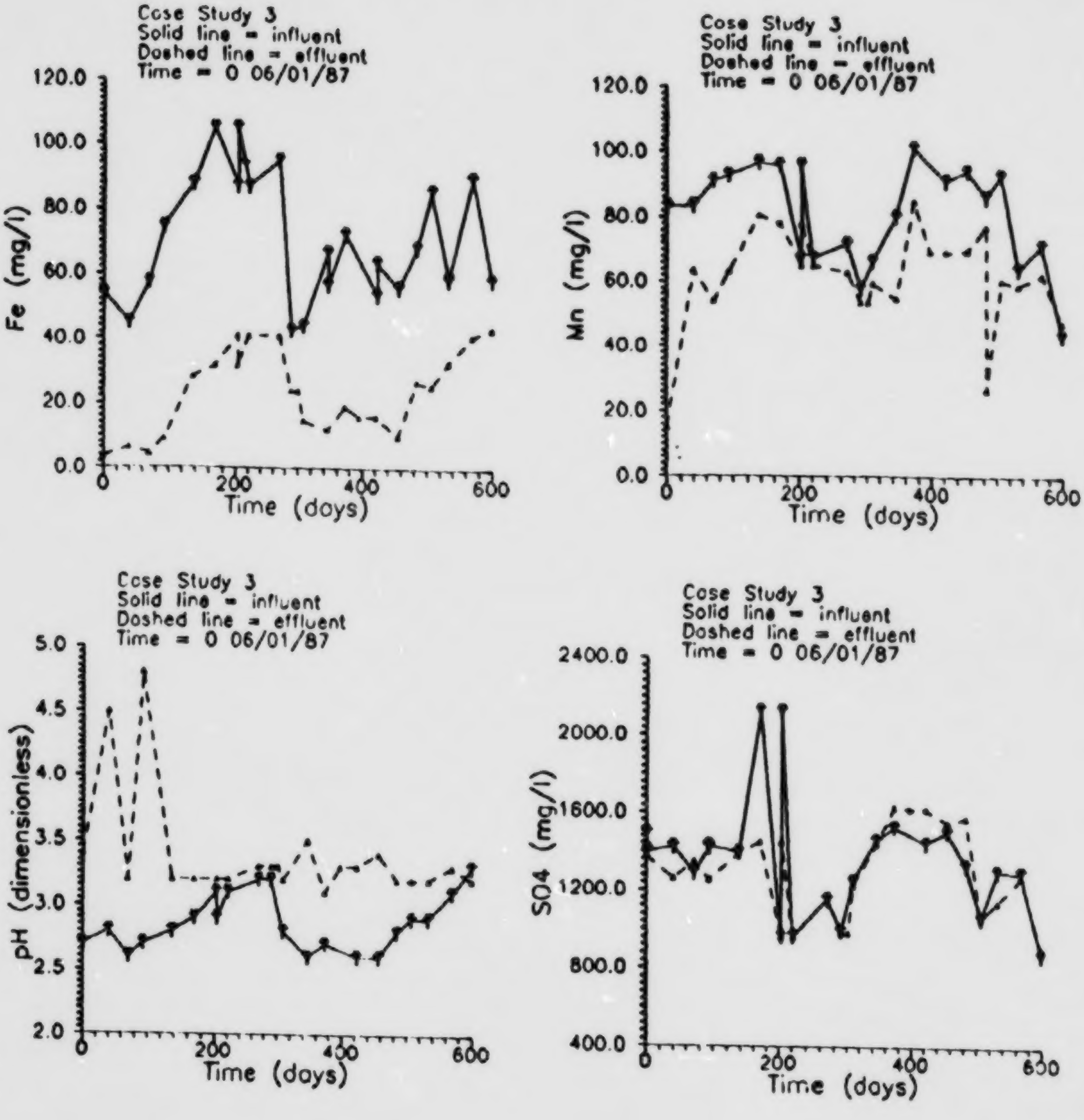

Fig. 6 The performance of a wetland as a function of time: Case Study 3. Note the seasonal variation in effluent Fe concentration.

OPERATION OF HOMESTAKE'S CYANIDE BIODEGRADATION WASTEWATER SYSTEM BASED ON MULTI-VARIABLE TREND ANALYSIS

Jim L. Whitlock[1] *and Greg R. Smith*[2]

ABSTRACT

Homestake's wasterwater treatment plant is based on biodegradation of cyanide species and bioadsorption of toxic heavy metals. Plant performance has continued to improve as measured by toxic component reduction in effluent concentration as well as efficiency and cost of operation. The plant has been in full scale operation since August 1984. A significant portion of the improvement may be attributed to natural evolution and self-regulation of the microflora. The remainder of the improvement is a result of modification of the physical plant and the utilization of trend analysis to facilitate process control. Data presented include loading rates, retention times, flow control, influent and effluent concentrations for parameters of interest, operational trends, permit limits, cost analysis and bioassay evaluation.

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[1] Chief Chemist Homestake Mining Company Lead, SD 57754

[2] Wastewater Treatment Plant Operator HMC Lead, SD 57754

LA BIODÉGRATION DU CYANURE DES EAUX USEÉS PAR L'INSTALLATION DE TRAITEMENT HOMESTAKE, BASÉE SUR L'ANALYSE DE LA TENDANCE MULTI-VARIABLE.

J. L. Whitlock[1] et G. R. Smith[2]

RÉSUMÉ

L'installation Homestake pour le traitement des eaux usées, est basé sur la biodégradation d'espèces de cyanure et par bio-adsorption de métaux toxiques lourds. Le rendement de l'installation a continué à s'améliorer comme en font foi la réduction des éléments toxiques mesurés dans la concentration des effluents ainsi que l'efficacité et les coûts d'exploitation. L'installation fonctionne à plein rendement depuis le mois d'août 1984. L'amélioration du rendement est attribuable en bonne partie à l'évolution naturelle et à l'autorégulation de la flore microbienne. Pour le reste, elle est attribuable à la modification de l'usine et à l'utilisation de l'analyse de la tendance facilitant la régulation du processus. Les données présentées comprennent les vitesses de chargement, les durées de séjour, la régulation du débit, les tendances opérationnelles, les restrictions du permis, l'analyse des coûts et l'évaluation de l'essai biologique.

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[1] Chef chimiste, Homestake Mining Company Lead, SD, É.-U.

[2] Opérateur de l'installation de traitement des eaux usées, HMC Lead, SD, É.-U.

INTRODUCTION

The Homestake Mine in Lead, SD treats and discharges up to 21,000 m^3 (5.5 MGD) of water per day. Wastewater sources include decanted water from Grizzly Gulch Tailings Impoundment and underground operations as deep as 2560 meters (8400 ft.). Discharge waters enter a cold water marginal trout fishery, Whitewood Creek, with immersion recreation permit limits. This discharge flow makes up to 56% of the total stream flow under low flow conditions. Prior to tailings impoundment and full scale start-up of Homestake's wastewater treatment plant (WWTP), Whitewood Creek was virtually void of all life forms for approximately 80 years. This receiving stream is now a small but excellent trout stream which recently yielded a state record trout.

Jim Whitlock, biochemist, and Terry Mudder, engineer, developed and designed a biological system for Homestake in 1981 after chemical detoxification schemes appeared to be unsatisfactory.

Treatment is based on degradation via biological pathways which remove free cyanide, thiocyanate, metal complexed cyanides and the cyanide degradation by-product, ammonia, through oxidation. Toxic heavy metals are adsorbed on the biofilm as described by Whitlock 1987, and Whitlock and Mudder 1985.

Pseudomonas sp. make up the predominant structure of the biofilm under attached growth conditions with the media base consisting of 48 Rotating Biological Contactors (RBC).

PLANT DESIGN

The theory behind plant design was to establish a system which would mimic natural surface biodegradation processes which were identified and quantified at Grizzly Gulch Tailings Impoundment. This entailed a design to "thin-film" 17,000-21,000 m^3 of wastewater under aerobic conditions over a short retention time. This thin film of wastewater would be required to contact sufficient biomass to accomplish cyanide degradation and metals adsorption. It was postulated that complete degradation of cyanide would produce ammonia and therefore a nitrification stage was proposed. RBC's and trickling filters were pilot tested, with RBC's selected as the most manageable technology.

These criteria were satisfied by a plant design (Figure 1), which incorporates 48 RBC's; 24 of which contain 9,290 m^2 (100,000 sq.ft) of surface area for cyanide degradation and metals removal and 24 RBC's with 13,935 m^2 (150,000 sq. ft.) utilized for nitrification. Supplemental air is added to the liquid phase of the RBC basins to maintain dissolved oxygen at 4.0 mg/L or higher. Biomass is attached to plastic media surface of the RBC in films which may approach 18,000 Kg per disk. Biomass weight on the disk is somewhat self-regulating by sloughing of the biomass. However, more turbulent aeration and pH shock have been employed on occasion to reduce biomass weights.

Pilot testing dictated a scheme based on a train of five disks; two located in a common basin for cyanide degradation/metals removal and three disks in a common basin with partition baffles to accomplish complete nitrification of ammonia (Whitlock 1987).

Effluent from basins is collected and routed to a clarifier where sloughed biomass from the disks is settled and surface clarified water is collected and filtered by dual media pressure sand filtration prior to discharge. A portion of the discharge flow is diverted to a bioassay toxicity testing facility where trout and other test organisms are utilized to evaluate effluent quality.

WASTEWATER CHARACTERIZATION

Tailings impoundment decant water and underground mine dewatering flows are the two major sources of wastewater to be treated. Contaminant concentrations fluctuate with seasons, impoundment freeze-over/turn-over, and mine sand backfill operations. Normal cyanidation water matrices are complicated by diesel fuel spills, degreasers, foaming lubricants, dispersants, biocides and various other chemical compounds finding use in modern mining methods. A basic wastewater characterization is presented in Table 1, and includes influent blend characteristics. The operators control the blend ratio of decant and mine water to set cyanide loadings for the plant as well as maintain reasonable temperatures. The plant operates efficiently at a temperature range of 10-30°C and recent research indicates that growth can be established at 5°C or lower. Results of this research will be presented in the very near future. Mine water temperature remains a relatively constant 26°C and decant water ranges from 0.05-22°C.

MICROBIOLOGY

Pseudomonas sp. are the predominant members of the biofilm and are responsible for the degradation of cyanide and bio-accumulation of metals. This system was designed to meet the following objectives; (1) provide near complete degradation and/or detoxification of thiocyanate, free cyanide and metal complexed cyanides (2) reduce heavy metal concentrations to trace levels via bioadsorptive mechanisms (3) convert ammonia to nitrate through the intermediate nitrite (4) convert sulfur from thiocyanate degradation to sulfate and (5) produce an aggressive system capable of withstanding shock loadings and other changes in wastewater characteristics and capable of rapid recovery from minor upsets. We preferred not to require additional carbon sources, additional chemical feeds and biological seedings. Biomass characterizations and biological degradation pathways are described in Whitlock 1987.

CHEMICAL ADDITIONS

The only chemical addition at present is 10 ml/min. of 87% Phosphoric acid which appears to be essential for energy storage and conversion in the nitrification stage. Influent phosphorous as P is elevated from 0.10 mg/L to 0.4 mg/L by this addition. On occasion, soda ash is added directly to the basin of an overweight disk at a level of 350 Kg per 115 m^3 water which elevates the pH to about 10.3 and causes rapid sloughing of a portion of the biomass.

LOADING RATES

Loading rates are based on cyanide and are seasonal. However, general loading rates may be expressed in kilograms/day/square meter of surface area for the RBC's. Load rates are as follows: (1) thiocyanate (SCN) 0.47 Kg/d/93 m^2 (1.05 lb/d/1000 sq.ft.), total cyanide (CNT) 0.06 Kg/d/m^2 (0.14 lb/d/1000 sq.ft.), Weak and dissociable cyanide (WAD) 0.03 Kg/d/93 m^2 (0.07 lb/d/1000 sq.ft.) ammonia as N, 0.11 Kg/d/m^2 (0.23 lb/d/sq.ft.) and heavy metals 0.02 Kg/d/93m^2 (0.04 lb/d/1000 sq.ft.).

Hydraulic retention times are 1.0, 1.5 and 3.0 hours respectively for basin 1 (first and second stage RBC's), basin 2 (third, fourth, and fifth stages), and the clarification/ filtration step.

PLANT PERFORMANCE AND OPERATIONAL TRENDS

Efficiency of removal rates for contaminants may vary with plant performance. However, a five-year analysis of daily data indicates removal rates of: thiocyanate 99-100%, total cyanide 96-98%, WAD cyanide 98-100%, copper 94-97% and ammonia conversion to nitrate 98-100% (Tables 2 and 3).

The order of ease of removal through cyanide degradation is free cyanide, thiocyanate, zinc cyanide, nickel cyanide, copper cyanide and iron cyanide from easiest to most difficult. Iron cyanide is only partially degraded and adsorption mechanisms play a strong role in removal of iron cyanide. Iron cyanide concentrations may be approximated by subtracting WAD cyanide from total cyanide concentrations for an individual influent or effluent sample. Figure 2 represents this comparison for iron attributed to iron cyanide vs iron in the influent, and figure 3 represents the same comparison for the effluent. Iron cyanide is removed to a greater degree than is iron in the system.

Metals adsorption is a function of biomass conditions as well as influent concentrations. Influent metals concentrations vary seasonally, being higher in winter and lower in summer as a result of cyanide concentrations and other factors in the tailings impoundment such as freeze-over. During periods of high biomass weight on the disks and low influent metals concentrations, metal removal rates may decrease and in some cases desorption will occur, resulting in effluent values which slightly exceed influent values (Figure 4).

Figure 5 represents a typical relationship between soluble and total copper; influent and effluent copper is adsorbed on solids and if solids are high (approaching 5-10 mg/l), in analytical samples they will be digested in total copper analysis. Total copper is a function of total suspended solids whereas soluble copper is not. It has been determined that ferric chloride at 5-10 mg/l could be added to form a ferric hydroxide floc and reduce soluble copper concentrations; however, it seems to have little effect on total copper.

Comparisons of total cyanide and WAD cyanide for influent (Figure 6) and effluent (Figure 7) have shown that WAD cyanide makes up a larger percentage of the total cyanide than it did when the plant was brought on line.

Total cyanide reduction in effluent has improved greatly in the plant as represented in a comparison of Figures 6 and 7 vs. Figures 8 and 9.

Total suspended solids in the effluent range from 1-10 mg/l and affect total copper concentrations (Figures 10 and 11) as well as total cyanide, because a portion of iron cyanide removal is based on adsorption in the solids.

Statistical analysis of data generated from full scale plants usually involves determining the effect of multiple dynamic variables rather than control of variables as in bench scale studies. Trend analysis seems to be an appropriate means of identifying variables of concern and increasing predictability for improved plant operation.

BIOASSAY EVALUATIONS

A bioassay facility is on line following the Treatment Plant and has been used to evaluate our program from pilot plant to full scale plant operation.

Extensive bioassay evaluation has yielded tests for trout for as long as 300 days without mortality and rigid examination of whole fish and fish tissue indicates the fish are in fine condition with accelerated growth rates due to higher metabolic rates in warm effluent waters as compared with receiving stream waters.

COST ANALYSIS

The total cost of Homestake's Wastewater Treatment Plant was approximately 10 million dollars or 2/3 of the projected cost of similar sized Hydrogen Peroxide plant. Daily operational costs (Table 4) are \$3.46 per kg (\$1.50 per pound) of cyanide degraded, \$0.55 per metric ton (\$0.44 per ton) of ore milled or about \$0.22 per M^3 (0.80 dollars per 1000 gal) of water treated.

CONCLUSIONS AND APPLICATIONS

Biological treatment seems to offer certain advantages over other cyanide destruction or detoxification methodologies. Advantages are (1) compatibility with receiving streams, (2) the ability to remain dynamic systems which adjust to changes in influent flows, (3) ability to recover from upset without human intervention, (4) treatment efficiencies which may exceed chemical treatment methods (5) lower operational cost than other detoxification technologies, (6) potential uses for conditions where electrical power does not exist and chemical additions and maintenance would be expensive or prohibitive.

Disadvantages are reported to be (1) the amount of research and time required for system design and start up, and (2) cold water temperature concerns. It appears that biological processes may work at lower temperatures than many believe and start up times can surely be shortened as an operational data base is developed.

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Whitlock, J. L., and Mudder, T. I., The Homestake Wastewater Treatment Process: Biological Removal of Toxic parameters from cyanidation wastewaters and bioassay effluent evaluation. Fundamental and Applied Biohydrometallurgy. Proceedings of the Sixth International Symposium on Biohydrometallurgy, Vancouver, B. C., Canada, August 21-24, 1985.

Table 1

WASTEWATER MATRIX mg/L

PARAMETER	DECANT WATER	MINE WATER	INFLUENT BLEND
Thiocyanate	110.00-350.00	1.00-33.00	35.00-110.00
Total Cyanide	5.50-65.00	0.30-2.50	0.50-11.50
WAD Cyanide	3.10-38.75	0.50-1.10	0.50-7.15
Copper	0.50-3.10	0.10-2.65	0.15-2.95
Ammonia-N	5.00-10.00	5.00-19.00	6.00-12.00
Phosphorous-P	0.10-0.20	0.10-0.15	0.10-0.15
Alkalinity	50.00-200.00	150.00-250.00	125.00-225.00
pH	7.00-9.00	7.00-9.00	7.50-8.50
Hardness	400.00-500.00	650-1400	500-850
Temperature °C	1.0-27.2	24-33	10.0-25

TABLE 2

INFLUENT, EFFLUENT AND PERMIT CONCENTRATIONS mg/L, 1988

	Thiocyanate	Total Cyanide	WAD Cyanide	Total Copper
Influent	62.0	4.1	2.3	0.56
Effluent	<0.05	0.07	0.02	0.07
Permit	1.00	0.10	0.13	

	TSS	Ammonia-N
Influent	—	5.60
Effluent	6.0	<0.50
Permit	10.0	1.0-3.9

TABLE 3

Effluent Total and WAD Cyanide concentrations mg/L

YEAR	TOTAL CYANIDE	WAD CYANIDE
1984	0.45	0.09
1985	0.37	0.05
1986	0.38	0.05
1987	0.22	0.05
1988	0.07	0.02

TABLE 4

Cost of Operation (U.S. Dollars)

	Per Kg CN Treated	Per Metric	Ton Per m^3 Eff
1984	11.79	1.20	0.20
1985	8.16	0.78	0.18
1986	5.51	0.60	0.12
1987	3.28	0.60	0.11
1988	3.46	0.55	0.11

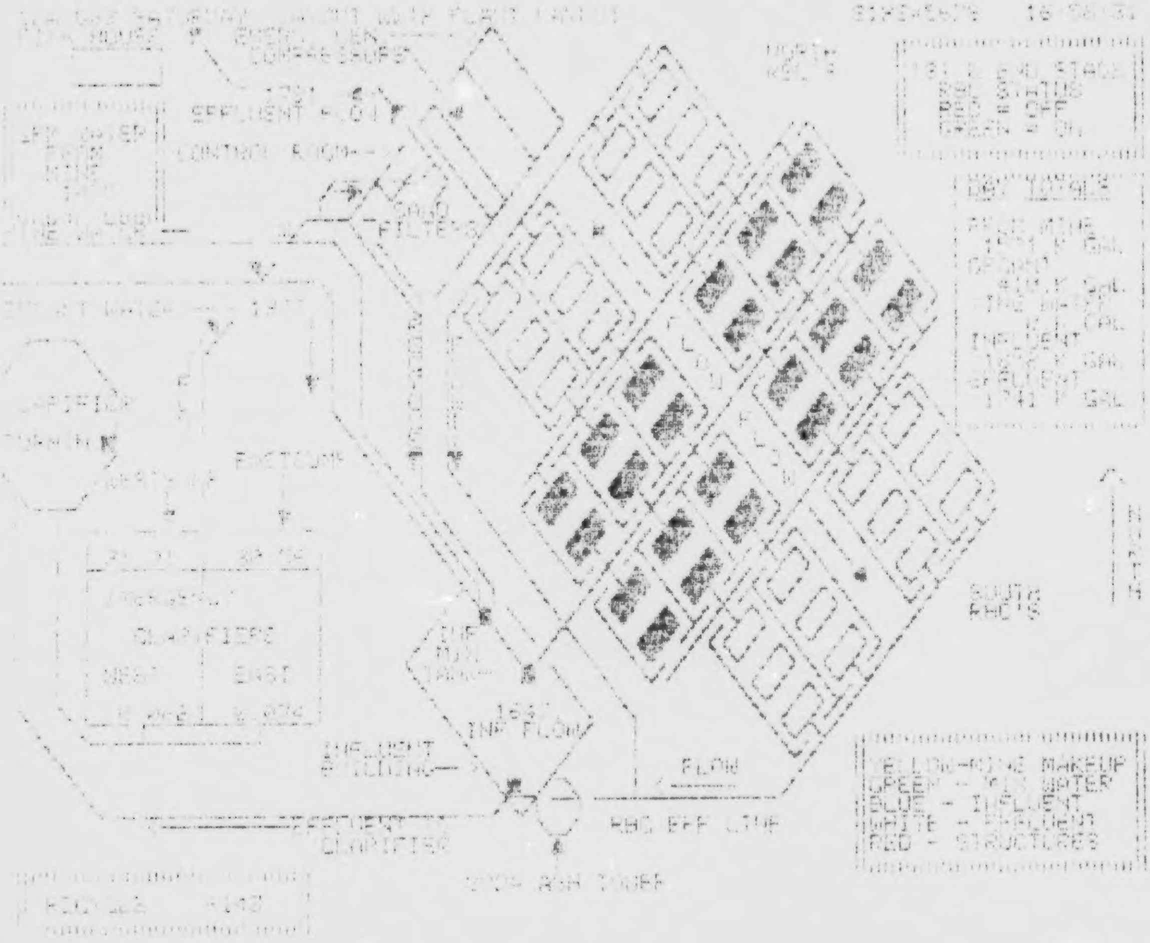
COMPRESSORS
EFFLUENT FLOW
CONTROL ROOM
SAND FILTERS
NORTH RBC'S
RBC STATUS
RED = OFF
GREEN = ON
INFLUENT
EFFLUENT
CLARIFIER
CLARIFIERS
WEST
EAST
INF MIX TANK
INF FLOW
INFLUENT BUILDING
FLOW
EFFLUENT CLARIFIER
RBC EFF LINE
SOUTH RBC'S
NORTH
YELLOW-MINE MAKEUP
GREEN - MIX WATER
BLUE - INFLUENT
WHITE - EFFLUENT
RED - STRUCTURES

Fig. 1 Waste Water Treatment Plant Layout

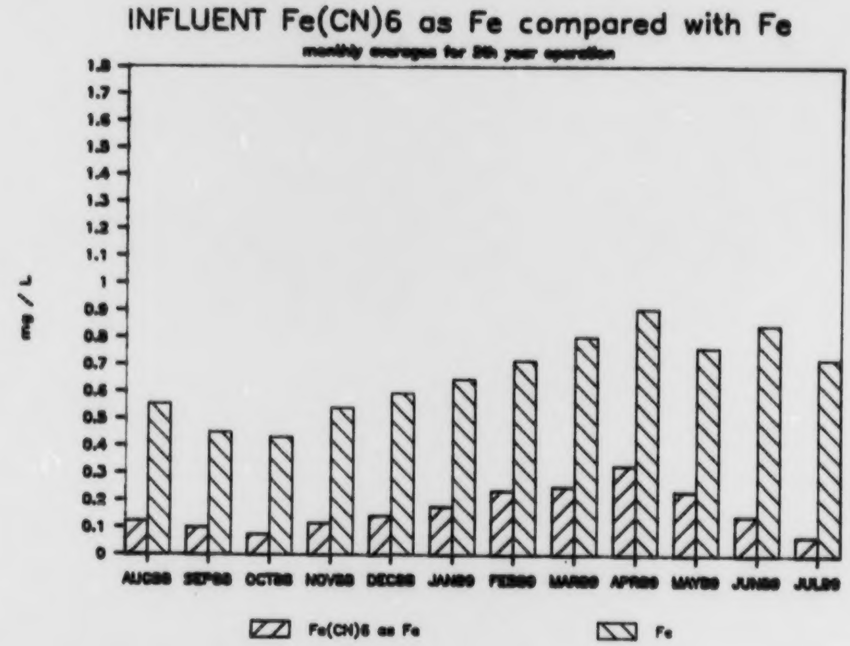

Fig. 2 INFLUENT Fe(CN)6 as Fe compared with Fe

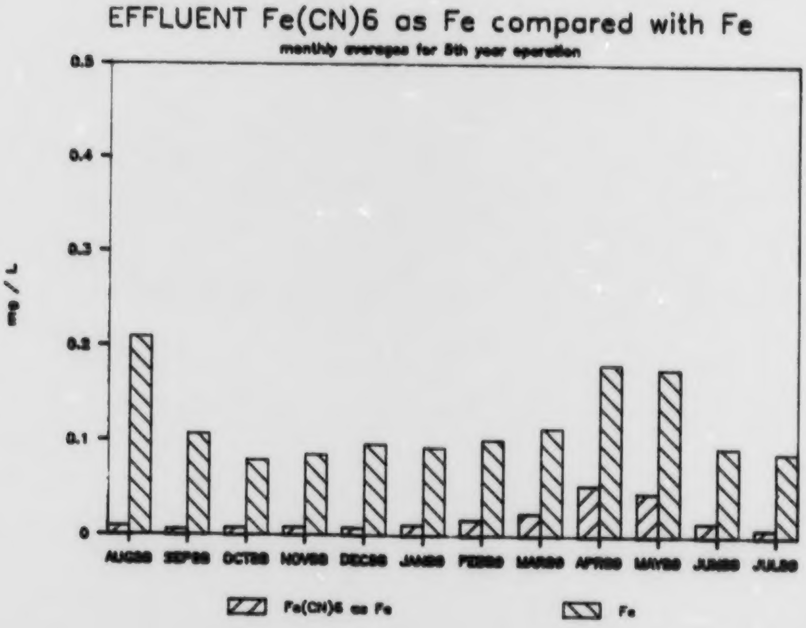

Fig. 3 EFFLUENT Fe(CN)6 as Fe compared with Fe

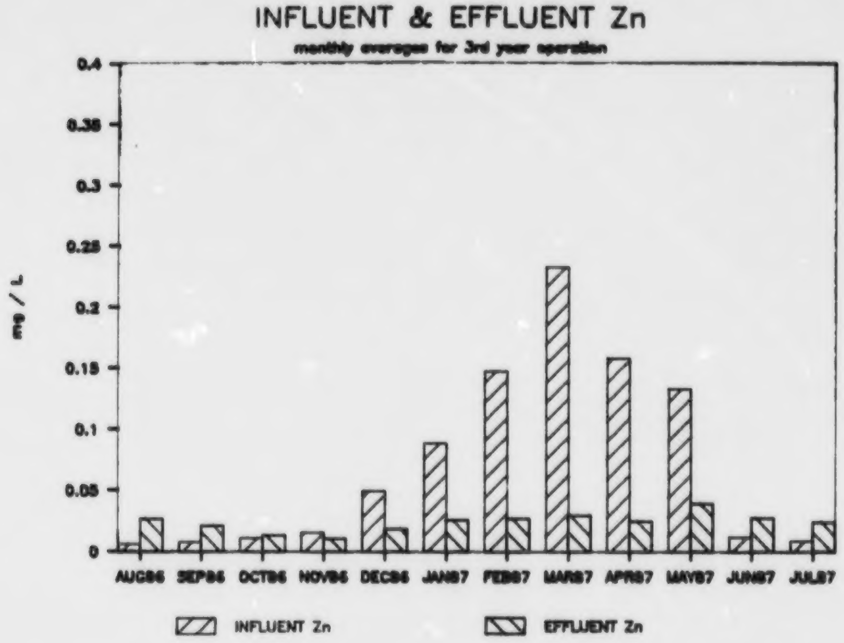

Fig. 4 INFLUENT & EFFLUENT ZN

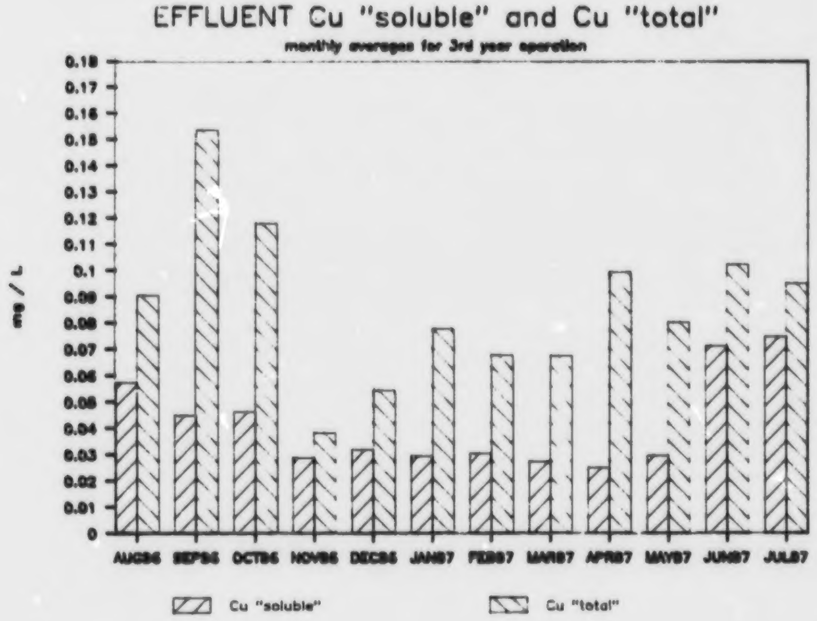

Fig. 5 EFFLUENT CU "soluble" and Cu "total"

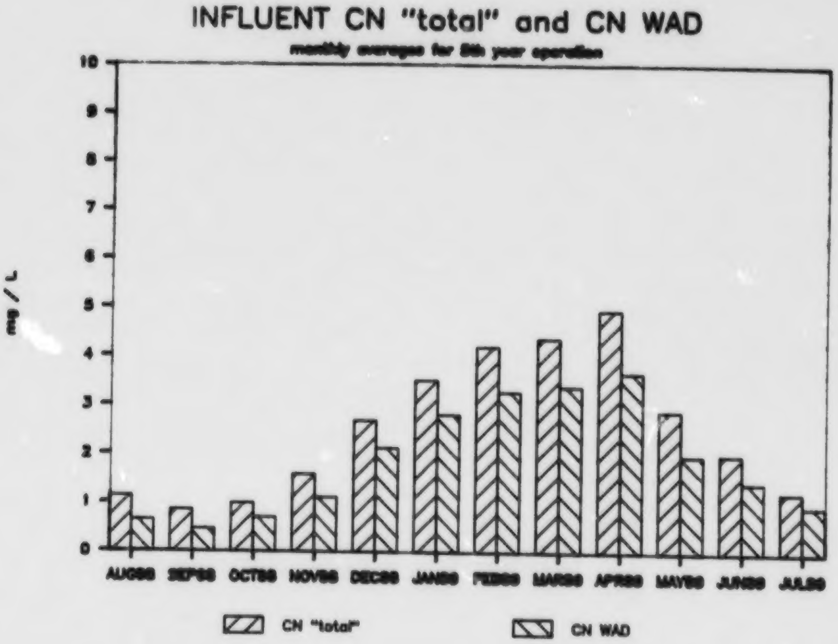

Fig. 6 INFLUENT CN "total" and CN WAD

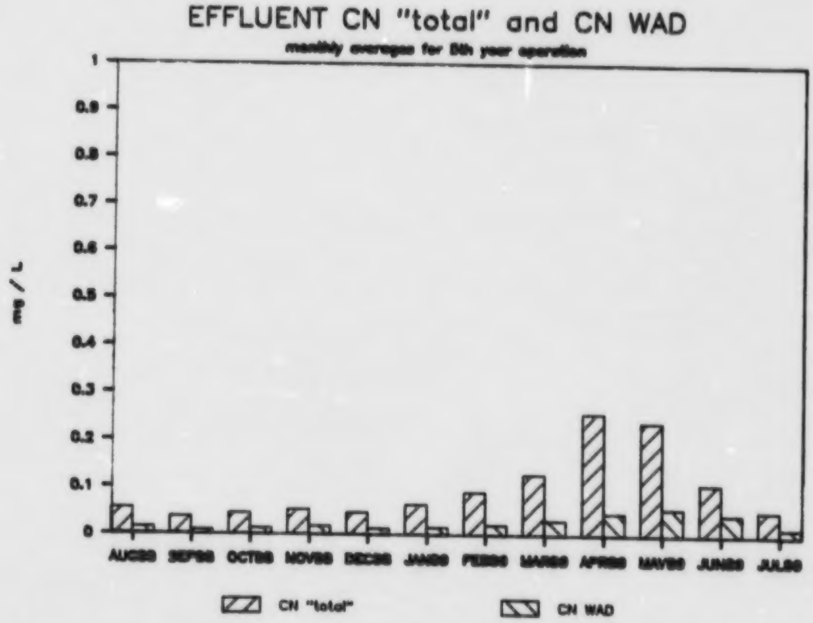

Fig. 7 EFFLUENT CN "total" and Cn WAD

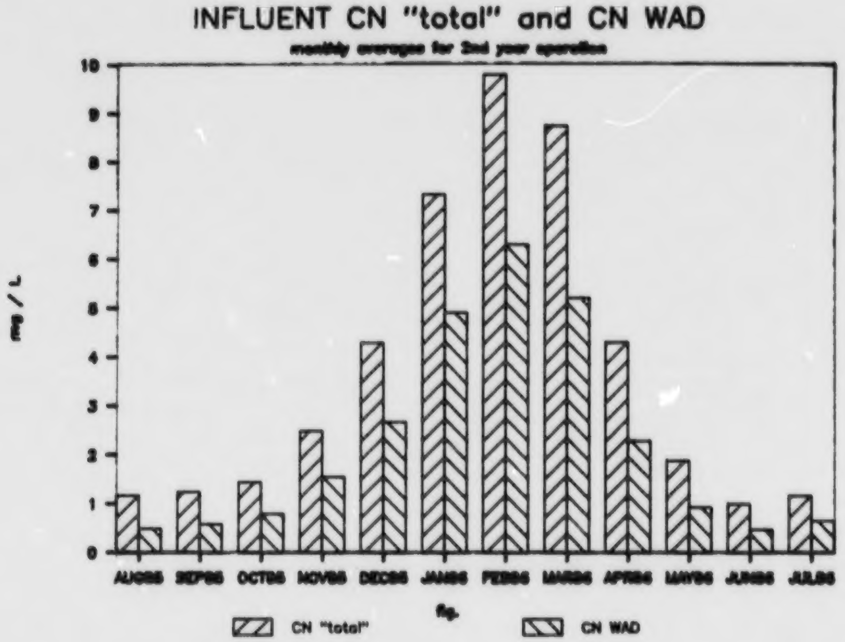

Fig. 8 INFLUENT CN "total" and CN WAD

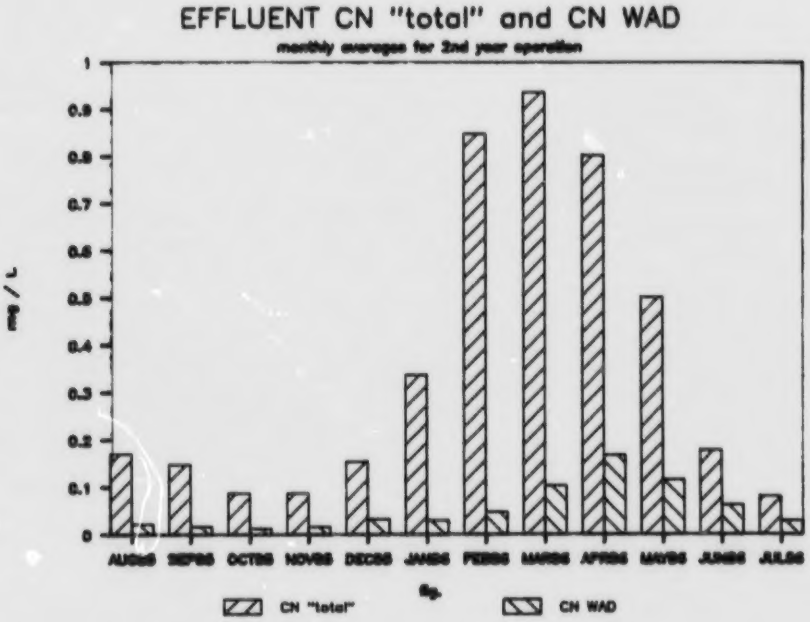

Fig. 9 EFFLUENT CN "total" and CN WAD

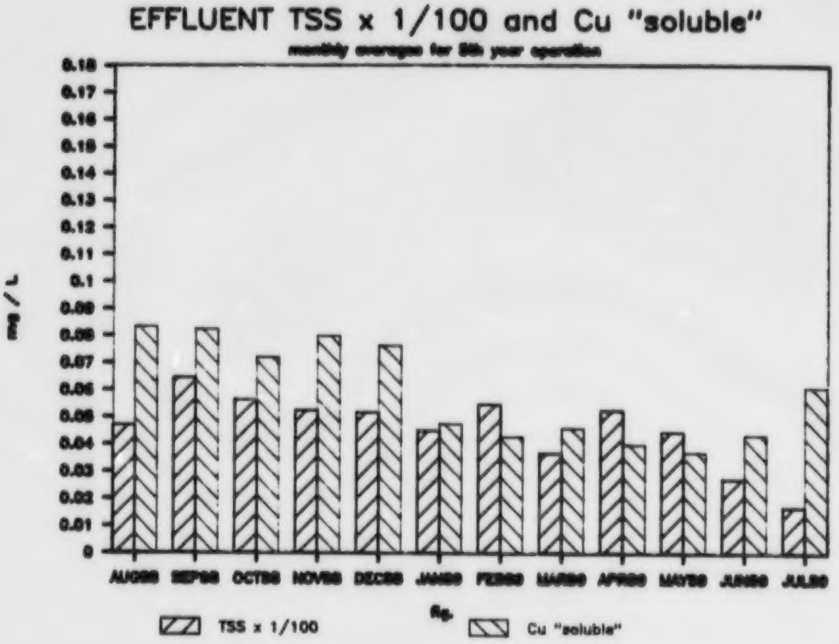

Fig. 10 EFFLUENT TSS x 1/100 and Cu "soluble"

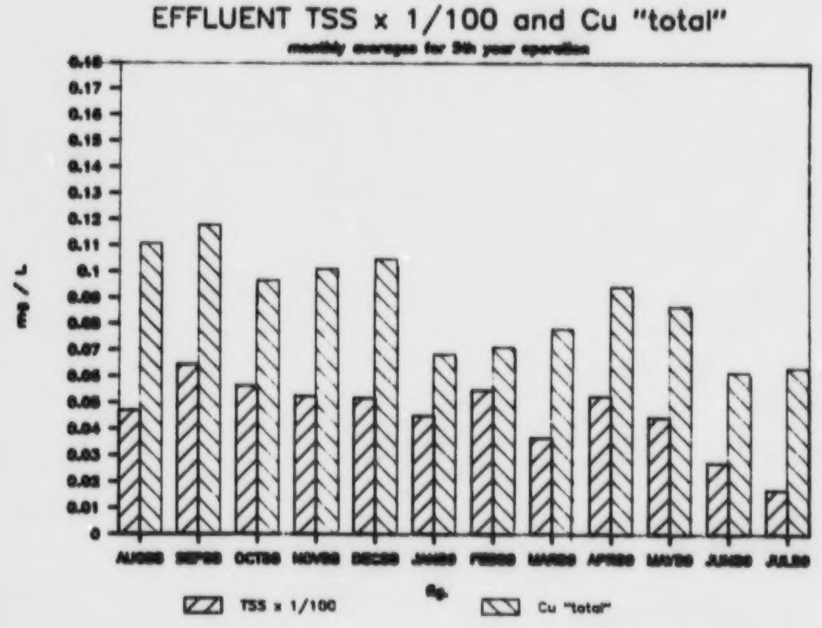

Fig. 11 EFFLUENT TSS x 1/100 and Cu "total"

APPLICATION OF ANAEROBIC PROCESSES IN CONSTRUCTED WETLANDS FOR THE TREATMENT OF ACID MINE DRAINAGE

By

Pamela E. McIntire and Harry M. Edenborn

U.S. Bureau of Mines Pittsburgh Research Center
Pittsburgh, Pa 15236

ABSTRACT

Bacterial sulfate reduction is a naturally-occurring process that proceeds only in the absence of oxygen and in the presence of sufficient organic carbon and sulfate. The net by-products of sulfate reduction, hydrogen sulfide and bicarbonate, can precipitate metal sulfides and neutralize acidic water, respectively. An experimental wetland was designed and built to maximize contact between mine drainage and the anaerobic zone of the organic substrate, where sulfate reduction takes place. Preliminary results from the wetland were encouraging. Iron concentrations decreased from 237 to 27 mg L^{-1}, pH increased from 2.9 to 6.5, and alkalinity increased from 0 to 1077 mg L^{-1} when the mine drainage was forced through the anaerobic zone. The observed improvement in water quality was due to both the inherent chemical characteristics of the organic substrate as well as bacterial activity. Bacterial sulfate reduction rates in the organic substrate ranged from 0.002 to 0.6 mmol/liter-sediment/day. Sulfate reduction rates are high enough to significantly affect the water quality of acid mine drainage. Minor modifications to existing wetland designs may improve the efficiency of water treatment by incorporating anaerobic bacterial processes.

UTILISATION DE PROCÉDÉS ANAÉROBIES DANS DES MARÉCAGES SERVANT AU TRAITEMENT DE DRAINAGE MINIER ACIDE

P.E. McIntire et H.M. Edenborn

Bureau des Mines des États-Unis, Centre de Recherche de Pittsburgh,
Pittsburgh, PA 15236 É.-U.

RÉSUMÉ

La réduction bactérienne du sulfate est un processus naturel qui se produit uniquement en absence d'oxygène, en présence d'une quantité suffisante de carbone organique et de sulfate. Le sulfure d'hydrogène et le bicarbonate générés suite à la réduction du sulfate, peuvent précipiter les métaux sulfureux et neutraliser l'eau acide. Un marécage expérimental a été crée et construit de façon a maximiser le contact entre le drainage minier et la zone anaérobie du substrat organique où le sulfate est réduit. Les résultats préliminaires se sont avérés encourageants. Les concentrations en fer ont baissées de 237 à 27 mg L^{-1}, le pH a augmenté de 2.9 jusqu'à 6.5 et l'alcalinité a augmenté de 0 jusqu'à 1077 mg L^{-1} suite au passage du drainage minier au travers de la zone anaérobie. L'amélioration de la qualité de l'eau observée, était due aux caractéristiques chimiques du substrat organique utilisé et également à l'activité bactérienne. Le taux de réduction bactérienne du sulfate dans le substrat organique se situait entre 0.002 jusqu'à 0.6 mmol/litre de sédiment/jour. Les taux de réduction du sulfate sont suffisamment élevés pour affecter le drainage minier acide. Des modifications mineures apporter à des marécages déjà existants qui permettent d'incorporer des procédés bactériens anaérobies, pourraient possiblement augmenter leur efficacité dans le traitement de l'eau.

INTRODUCTION

At over 300 mine sites in the bituminous coal region of the eastern United States, acid mine drainage (AMD) is being treated biologically using constructed wetlands (Hedin, 1989). In general, the processes at work in these systems are aerobic. The oxidation of ferrous to ferric iron and the subsequent precipitation of iron oxyhydroxide floc, for example, are dominant processes:

$$Fe^{2+} + 0.25O_2 + 1.5H_2O \rightarrow FeOOH\ (solid) + 2H+ \qquad (1)$$

Ferrous iron tends to autooxidize in aerated solution at pH greater than 6, while in more acidic water naturally-occurring bacteria catalyze the reaction (Singer and Stumm, 1970; Ehrlich, 1981). Although iron oxidation and hydrolysis processes are effective at removing much of the iron from AMD, these processes do nothing to help raise the pH of the water or decrease the acidity. In fact, the pH of water is actually lowered by these reactions (Equation 1). Many constructed wetlands with circumneutral pH and iron-contaminated inflow water produce water with a lower pH (Brodie *et al.*, 1988; D. Kepler, pers. commun., 1989).

Ironically, bacterial processes capable of increasing the pH and alkalinity of AMD entering constructed wetlands are already found there, but current wetland designs do not take advantage of them. Probably the most useful of these processes for treating AMD is bacterial sulfate reduction, a naturally-occurring process that proceeds in many environments in the absence of oxygen and in the presence of suitable organic substrates and sulfate. Sulfate-reducing bacteria use organic carbon and sulfate in the process of anaerobic respiration:

$$2CH_2O + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \qquad (2)$$

The reaction has promise in the treatment of acid and metal-contaminated mine waters because the by-products of the reaction, hydrogen sulfide and bicarbonate, can be used to precipitate many metals and raise the pH of the water, respectively. Bacterial sulfate reduction is very common in estuarine and marine sediments where all the necessary ingredients are abundant. The process is less common in freshwater systems, where there are typically low concentrations of sulfate (Postgate, 1984). However, the pyrite oxidation reaction that creates AMD also produces sulfate in large amounts. When AMD percolates into stream bottoms or constructed wetlands containing abundant organic matter and debris, bacterial sulfate reduction can proceed. Wetlands constructed to treat AMD are generally built to include a large amount of organic matter, and anaerobic conditions develop rapidly within that material. Calculations using data from natural wetlands and constructed wetlands have suggested that the use of a sulfate reduction treatment system is theoretically feasible (Hedin, Hammack, and Hyman, 1988). In the present study, we present data collected from a constructed wetland designed to incorporate bacterial sulfate reduction as a treatment process.

MATERIALS AND METHODS

An experimental wetland was constructed at the Friendship Hill National Historic Site, located in Fayette County, Pa. AMD flows into the wetland at its southernmost point at site WSO1 (Fig 1a). The water has a pH of about 2.5, total iron concentration of 50 to 250 mg L^{-1}, and a sulfate concentration of 1000 to 2500 mg L^{-1}. The constructed wetland is divided into 3 cells. The first cell is a holding pond that does not treat the AMD. The remaining two cells are each divided into three different treatment lanes separated by fibreglass sheeting. In cell 2, the AMD flows across the surface of the organic substrate, but in cell 3, subsurface infusion pipes can be used to carry AMD down to the bottom of the organic substrate layer (Fig. 1b).

The six treatment lanes were each constructed with a 15 cm (6 in.) layer of gravel on the bottom, covered by a 46 cm (18 in.) layer of composted organic substrate. The organic substrate used was discarded mushroom compost obtained from the Moonlight Mushroom Corporation of Worthington, PA. Whole cattail plants were transplanted to all cells and the compost was flooded with 8 to 20 cm

(3-8 in.) of water. The basal gravel layers in the cells consisted of crushed limestone in lanes B and C, and a non-alcareous river gravel in lane A.

Surface water samples were collected from nine locations in the wetland biweekly for eight months. pH measurements in the field were made using an Orion SA230 portable pH meter. Alkalinity (as $CaCO_3$ equivalent) was determined by titrating a 50 mL sample with 0.2 N H_2SO_4 to pH 4.8. Acidity was measured in a 50 mL sample that was first boiled with 1 mL of 30% H_2O_2 and then titrated with 0.1 NaOH to pH 8.3. Total iron concentrations were determined for acidified water samples using an ICP atomic emission spectrophotometer, following sample filtration through a 0.45 μm membrane filter. Sulfate concentrations were determined by barium chloride titration, using thorin as the end point indicator.

Platinum electrode potentials in organic substrate cores taken from the wetland were measured within 20 min of core removal. Measurements were made at 4 cm intervals throughout each core. A platinum wire was inserted through a silicone plug located at each sampling interval and a reference electrode was suspended in the surface water overlying the substrate. The wire and electrode were attached to a voltage meter standardized using Zobell's solution (Eh = +430 mV) prior to each measurement series.

Sediment sulfate reduction rates and total reduced sulfur concentrations were determined using the method of Herlihy and Mills (1985), which is a modification of the method of Jorgensen (1978). Vertical cores of organic substrate from the wetland were transported to the laboratory within 8 h of their removal. Five to 8 mL subcores were taken from two depths in each core. The subcores were immediately injected with 50 μL of carrier-free ^{35}S-labelled Na_2SO_4 (5 mCi/mL; New England Nuclear). Subcores were incubated at in-situ temperature for 2 to 14 d. Incubation times were chosen to allow 5 to 50% of the labelled sulfate to be converted to sulfide. After incubation, the subcores were frozen and stored at -4%C prior to analysis. The amount of labelled sulfide formed during incubation was determined by acid distillation of the subcores and the collection of the released hydrogen sulfide in traps containing a solution of 10% NaOH. Total reduced sulfur concentrations reported in this paper consist of the sum of an acid volatile sulfide (AVS) fraction (hydrogen sulfide, bisulfide ion, and iron monosulfide) and a chromium reducible sulfur (CRS) fraction (elemental sulfur and pyrite). Sulfate reduction rates were determined as described by Jorgensen (1978).

Interstitial porewater samples were collected using equilibrators similar to those designed by Hesslein (1976). These equilibrators consist of thick Plexiglas stakes containing wells of approximately 30 mL volume spaced 2 cm apart along the length of the stake. The wells were filled with deionized deoxygenated water and covered with a 0.2 μm membrane filter sheet. The apparatus was pushed down into the organic substrate of the wetland and allowed to equilibrate with the interstitial water for 3 to 4 weeks. Ten mL samples were then extracted from each well and placed in vials containing 1 mL of 2 N zinc acetate. Sulfate concentrations in these samples determined by liquid chromatography were used in the calculation of sulfate reduction rates.

The percent volume of water in subcores used to measure sulfate reduction rates was determined by measuring the difference in weight of similar subcores before and after drying, and assuming the density of water to be equal to 1 g/cm^3.

RESULTS AND DISCUSSION

Experimental Wetland

Study of the Friendship Hill constructed wetland showed that increasing contact between AMD and the anaerobic zone of the organic substrate improved the quality of the water leaving the wetland. When subsurface infusion pipes were turned on and the AMD was forced down into the anaerobic substrate, an immediate increase in pH and alkalinity and a net removal of total iron were observed

(Fig. 2, 3 and 4). During this time, the pH of the outflow water increased to as high as 6.5, the alkalinity increased up to 500 mg L^{-1}, and the total iron concentration decreased from 200 to 20 mg L^{-1}. In contrast, little improvement in water pH or alkalinity was observed when the AMD only flowed across the surface of the organic substrate. When the subsurface infusion pipes were turned off, the observed improvements in water quality disappeared.

It is probable that much of the initially observed improvement in water quality reported here was due to the chemical characteristics of the spent mushroom compost used to construct the wetland. The neutralization potential capacity of fresh mushroom compost is about 3.5% $CaCO_3$ equivalents (Hammack and Hedin, 1989). If we consider that approximately 32,000 kg (35 tons) of compost were placed in each lane containing a subsurface drainage system, and know that approximately 3.3 10^5 liters of AMD of average composition (1000 mg L^{-1} acidity) passed through the compost during 31 d of operation, approximately 30% of the total initial alkalinity of the compost would have been exhausted. If all of the alkalinity could be used to treat AMD before it left the wetland, chemical neutralization might be expected to continue for three months. However, this assumes that the water flows through the wetland in such a way that all compost is contacted equally and no portion of the compost is exhausted of its neutralization potential before any other. In reality, flow patterns are likely to occur that use up the neutralization potential of some compost and result in less effective neutralization of the AMD. In addition, bacterial sulfate reduction can be expected to contribute neutralization potential to the compost with time, adding to the total pool of alkalinity. The potential significance of this biological process is addressed later in this section.

The first time that underflow drains were turned on in the Friendship Hill wetland, there were large differences in the chemical composition of water flowing from the lanes containing either calcareous or noncalcareous gravel. The pH, alkalinity, and total iron concentration in the treated water were all significantly improved in the lanes containing the calcareous gravel (Fig. 2, 3 and 4). This suggests that dissolution of the limestone initially played a major role in improving the water quality. However, the second time the subsurface infusion pipes were turned on, the limestone gravel lanes were just as effective as those containing noncalcareous gravel. Although it was hoped that the anaerobic environment established in the organic substrate of the wetland would prevent the armouring of the gravel with iron oxyhydroxides and/or gypsum, it appears that this probably occurred anyway. This explanation seems reasonable, considering that the surface water introduced to the gravel layers contained both oxygen and ferric iron. These data imply that there is no significant advantage in using limestone gravel in the construction of wetlands employing anaerobic systems, in agreement with the laboratory results of Hammack and Hedin (1989).

Soon after the subsurface infusion pipes were turned on for the second time at Friendship Hill, they became clogged with iron oxyhydroxide floc and organic debris. An alternative method of introducing AMD to the anaerobic regions of the wetland without the use of pipes was then devised. This method involved the installation of a sheet of Plexiglas in a test lane of the wetland, places so that water in the lane was dammed behind the sheet and was forced to flow downward and beneath the dam. This is somewhat analogous to the hay bale dams found in some wetlands. Water downstream of these dams often has a higher pH (Hedin *et al.*, 1988; Stark *et al.*, 1988), although this is in part due to sulfate reduction activity occurring within the hay bales themselves. In the Plexiglas dam method used in the Friendship Hill wetland, the dammed water was forced to make contact with the basal gravel and anaerobic compost layers. Initially, water quality improvements were observed again (Fig. 2, 3 and 4); however, after a few weeks of operation the water quality was no longer affected positively by the dam system.

Measurements of pH in the porewater of the organic substrate at the Friendship Hill wetland showed that the pH generally remained between 6.0 and 7.0, even though the surface water usually had a pH of 2.5 to 3.5. This high porewater pH most likely reflects the influence of alkaline substances present in the compost as well as those produced by the natural bacterial population. When the Plexiglas dam was used, the increased flow of AMD through the relatively small volume of compost located on either side of the dam exhausted the neutralization potential of the compost. Porewater pH values deep in the compost above the dam after its operation were all low (3.2 to 4.5), supporting this hypothesis.

Sulfate Reduction Rates

During the course of this study, the redox potentials measured below 15 cm depth in the organic substrate of the constructed wetland decreased steadily (Table 1). This is an indication of the gradual exhaustion of oxidized chemical species, the accumulation of reduced species, and the activity of sulfate-reducing bacteria.

Sulfate reduction rates measured in the organic substrate of the Friendship Hill wetland were highly variable, reflecting the heterogenous nature of the organic compost (Fig. 5). Maximum detectable rates of sulfate reduction activity during the study period were high for freshwater environments, however, and are more comparable to rates found in shallow coastal marine sediments (Skyring, 1987) and a freshwater lake that receives AMD (Herlihy and Mills, 1985). Sulfate reduction rates remained consistently low throughout the winter but increased as the summer progressed, consistent with the predicted overall increase in biological activity with seasonally higher temperatures (Fig. 5). The concentration of total reduced sulfur compounds also increased in the wetland substrate with time (Fig. 6). This confirms that the by-products of bacterial sulfate reduction do accumulate in the wetland substrate and can probably be used to affect the quality of AMD under properly engineered conditions.

It is of interest to consider the potential role of bacterial sulfate reduction in the neutralization of AMD flowing through a wetland constructed to incorporate this process as a treatment method. If the maximum sulfate reduction rates measured in the Friendship Hill wetland were maintained throughout the entire volume of organic substrate for six months, and AMD chemical composition and flow rates were as described previously, 17% of the AMD flowing through the wetland during that time could be treated by bacterial activity alone. This percentage could be increased significantly by enhancing the sulfate reduction rate or by lowering the flow rate of AMD through the system. Even at low rates, bacterial sulfate reduction will lengthen the period of time that AMD can be fully neutralized, until the alkaline components in the substrate are exhausted. It will require additional study to determine if higher rates of sulfate reduction can be stimulated and maintained by improving wetland design and construction practices.

CONCLUSIONS

Although it has been known for several years that bacterial sulfate reduction takes place in wetlands constructed for the treatment of acid mine drainage, the rate measurements reported here represent the first to be published in the literature to our knowledge. The study has found that sulfate reduction occurs at relatively high rates, especially for freshwater systems, but that these rates are highly variable in the heterogeneous environment of the wetland organic substrate, and they vary seasonally. It is apparent from laboratory and field studies that this process may prove to be useful in the treatment of acid and metal mine drainage if properly engineered systems can be designed. However, the problem of designing and constructing an effective system incorporating bacterial sulfate reduction for the long-term treatment of mine drainage remains to be solved.

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Table 1

Redox potentials determined with a platinum electrode at depths below 15 cm in the substrate of the Friendship Hill wetland

Date	Platinum Electrode	Potential (mV)
	Min	Max
11/01/88	32	449
11/15/88	-18	238
12/16/88	-84	33
01/31/89	-46	-10
05/31/89	-108	-9

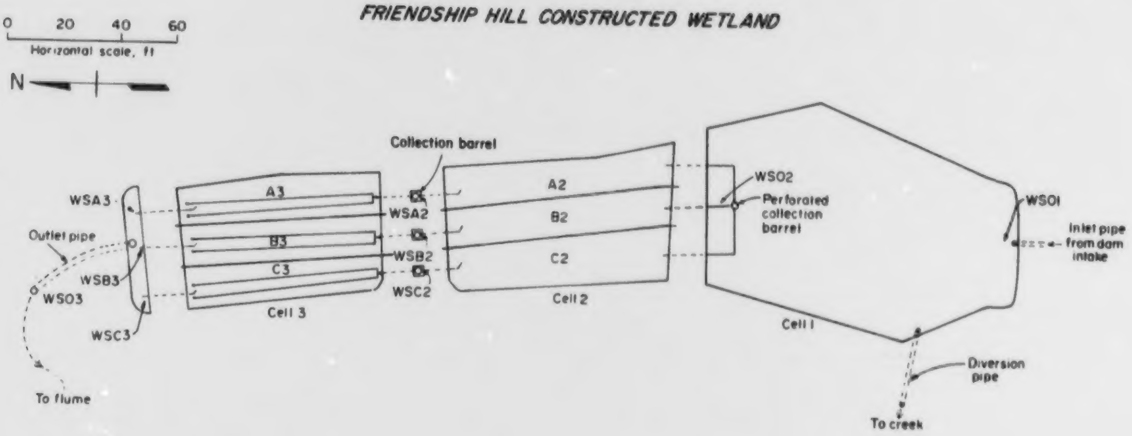
FRIENDSHIP HILL CONSTRUCTED WETLAND
0
20
40
60
Horizontal scale, ft
N
Collection barrel
WSA3
Outlet pipe
WSB3
WSO3
WSC3
To flume
A3
B3
C3
Cell 3
WSA2
WSB2
WSC2
A2
B2
C2
Cell 2
WSO2
Perforated collection barrel
WSO1
Inlet pipe from dam intake
Cell 1
Diversion pipe
To creek

Fig. 1a Plan view of Friendship Hill constructed wetland.

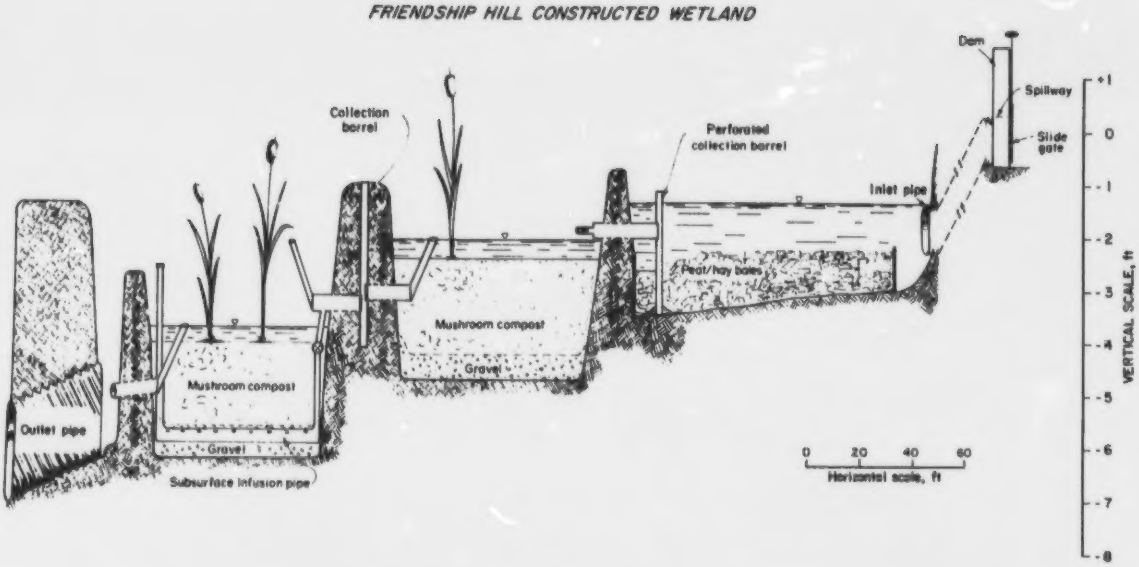
FRIENDSHIP HILL CONSTRUCTED WETLAND
Dam
Spillway
Slide gate
Collection barrel
Perforated collection barrel
Inlet pipe
Peat/hay bales
Mushroom compost
Gravel
Mushroom compost
Gravel
Outlet pipe
Subsurface infusion pipe
0
20
40
60
Horizontal scale, ft
+1
0
-1
-2
-3
-4
-5
-6
-7
-8
VERTICAL SCALE, ft

Fig. 1b Typical longitudinal cross-section of the Friendship Hill constructed wetland.

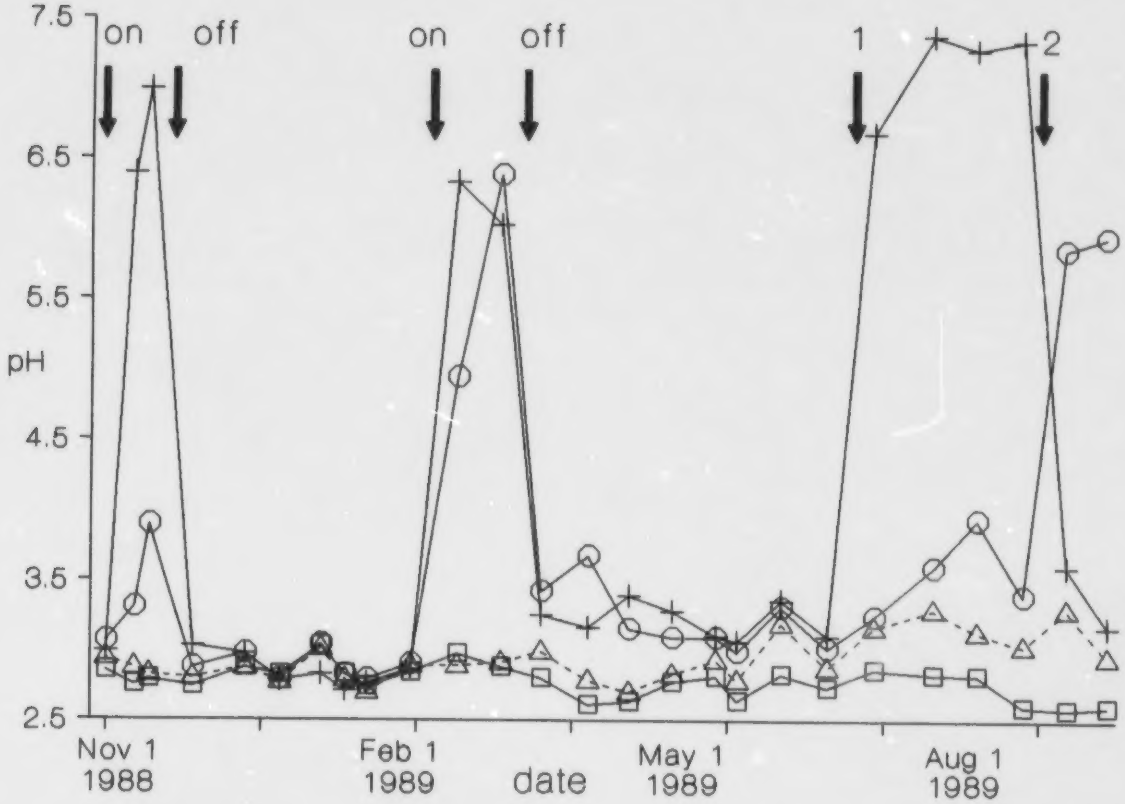

Fig. 2 The pH of water in the Friendship Hill constructed wetland system during the study period. Symbols indicate: untreated water flowing into the wetland (open squares); water leaving surface flow only cell in wetland (open triangles); water leaving subsurface flow wetland cell containing noncalcareous gravel (open circles); water leaving subsurface flow wetland cell containing limestone gravel (plus signs). Arrows mark times when the subsurface system was turned on and off. Symbols "1" and "2" denote when underflow dams were installed in the calcareous and noncalcareous gravel lanes, respectively.

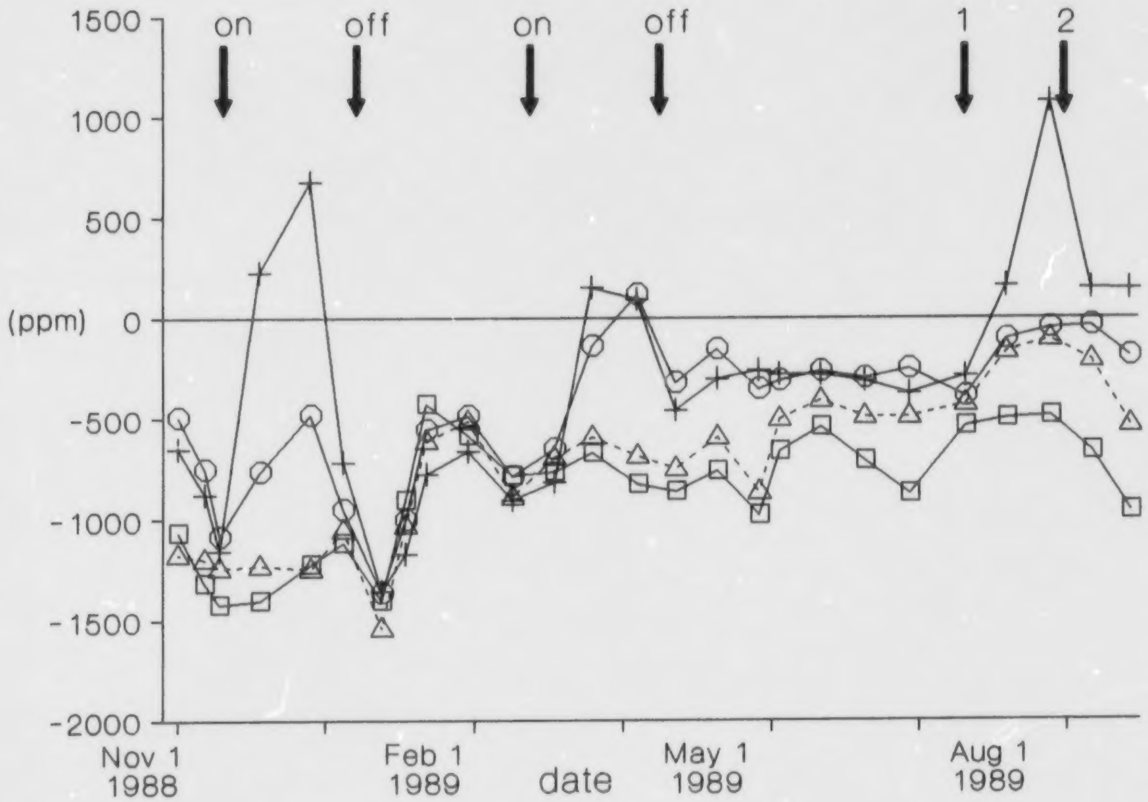

Fig. 3 Alkalinity (as ppm $CaCO_3$) of water in the Friendship Hill wetland system during the study period. Symbols are the same as described for Fig. 2.

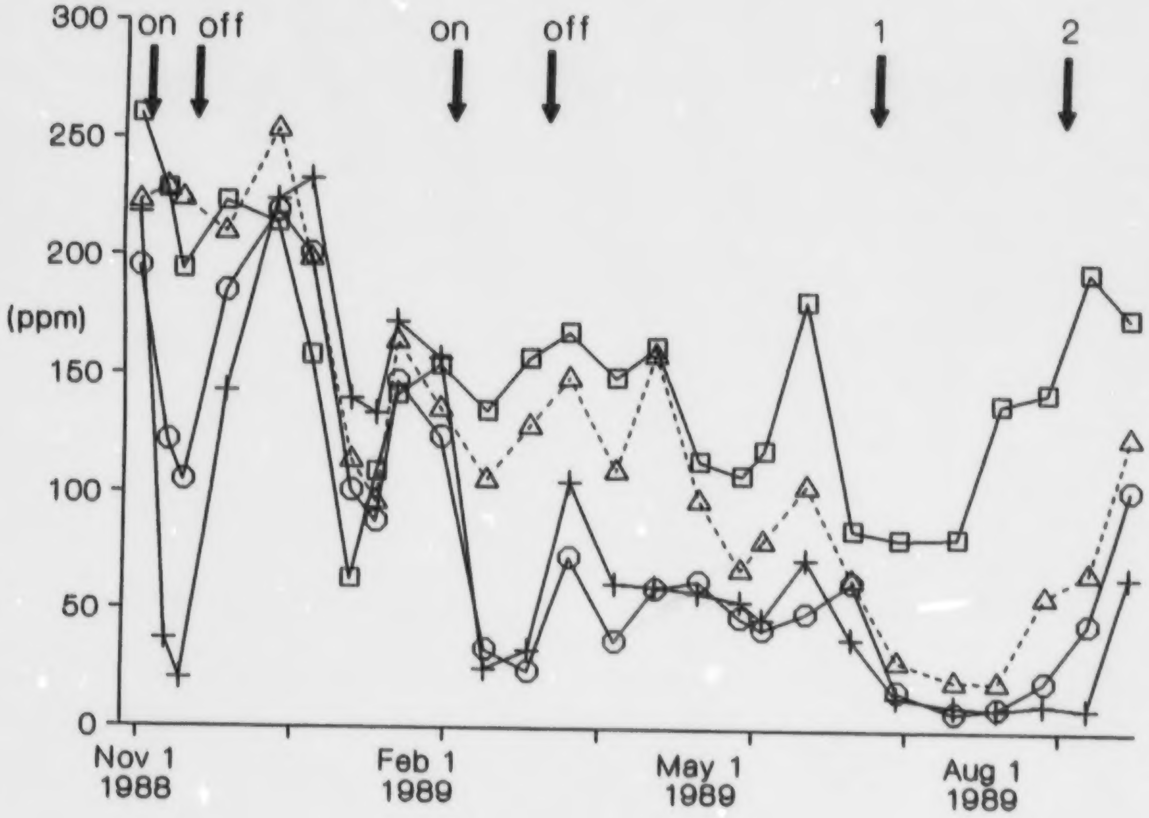

Fig. 4 Total iron concentrations in water in the Friendship Hill wetland system during the study period. Symbols are the same as described for Fig. 2.

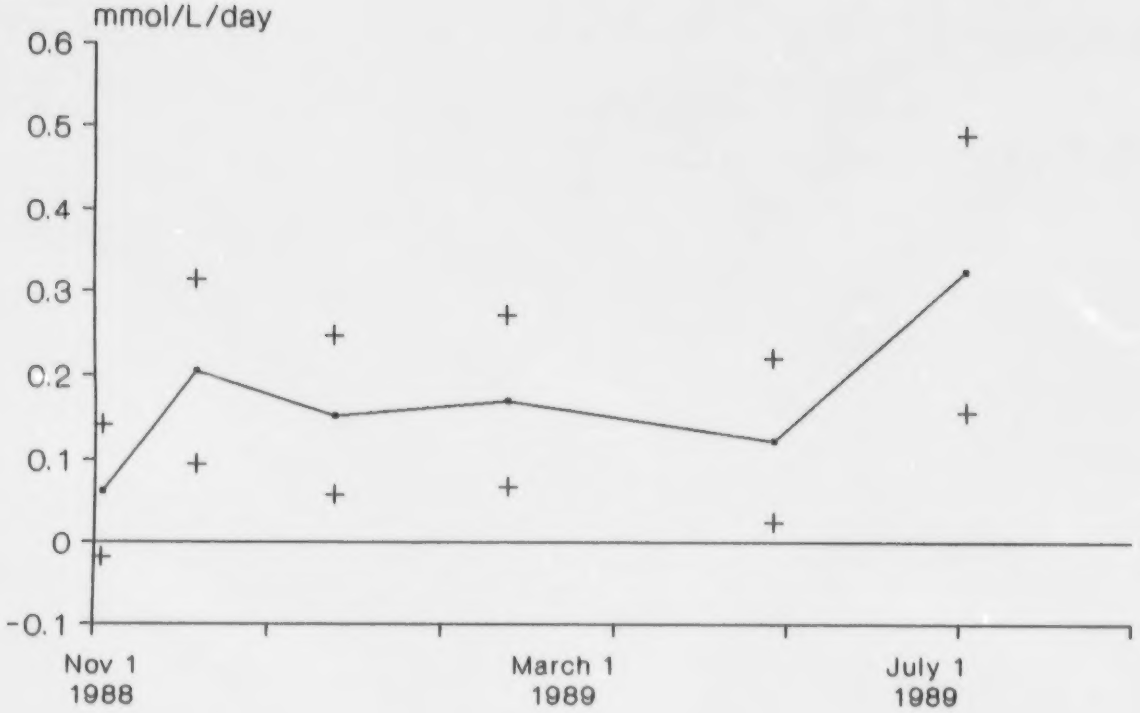

Fig. 5 Sulfate reduction rates measured in the organic substrate of the Friendship Hill wetland. x + SD is shown for each sampling date (n = 10 to 40).

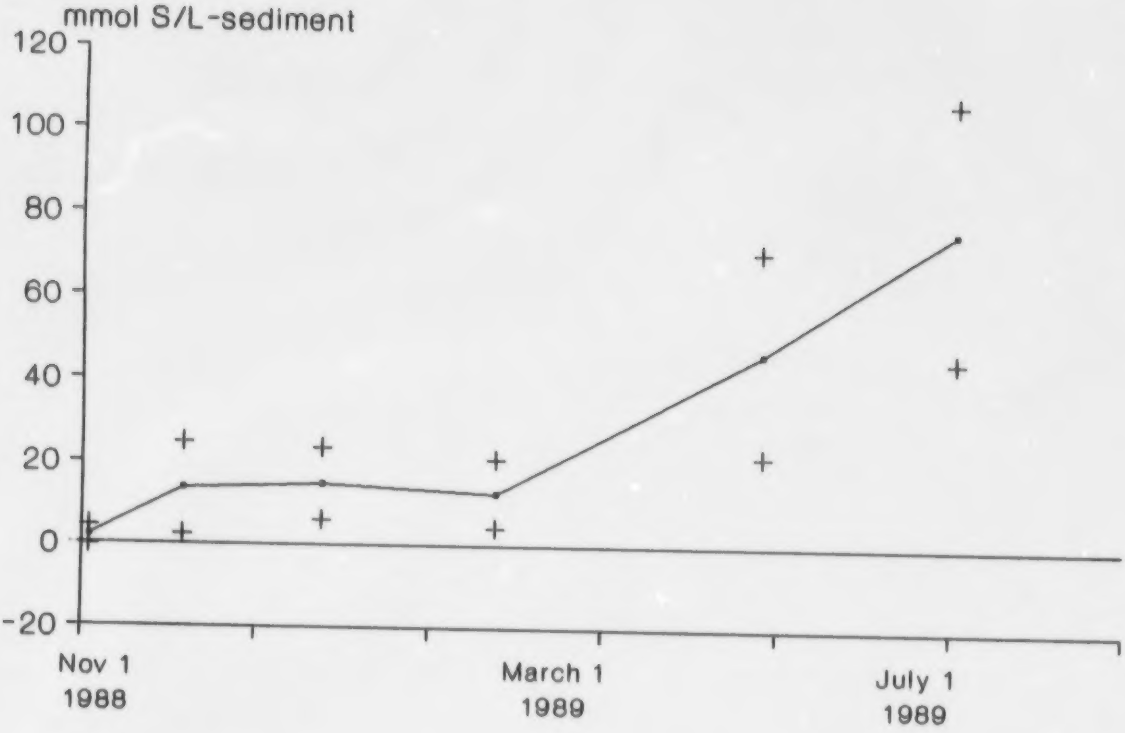

Fig. 6 Total reduced inorganic sulfur concentrations in the organic substrate of the Friendship Hill wetland. x + SD is shown for each sampling date (n = 10 to 40).

BENCH-SCALE PROCESS DEVELOPMENT OF SELENIUM REMOVAL FROM WASTEWATER USING FACULTATIVE BACTERIA

by

P. B. Altringer
Supervisory Metallurgist

D. M. Larsen
Chemical Engineer

K. R. Gardner
Physical Science Technician Salt Lake City Research Center Bureau of Mines U.S. Department of the Interior Salt Lake City, UT

ABSTRACT

The Bureau of Mines, U.S. Department of the Interior, is conducting laboratory research on bacterial removal of selenium from wastewaters. A mixed bacterial culture has been removing up to 96% of the selenium from agricultural drainage waters in a small-scale, continuous, plug-flow reactor for 1 yr; selenium removal is correlated to changes in the bacterial cultures. Data are presented on the complex interrelationships between the reduction of selenate and other compounds such as nitrate, phosphate, and sulfate. Bacterial respiration studies, results from X-ray diffraction studies, and scanning electron photomicrographs of the product are presented to further elucidate the mechanisms of selenium removal. Results also show that bacteria isolated from agricultural drainage waters can be applied successfully to remove selenium from mine tailings pond water.

MISE AU POINT D'UN PROCÉDÉ EXPÉRIMENTAL À PETITE ÉCHELLE POUR L'EXTRACTION DU SÉLÉNIUM DES EAUX USÉES AU MOYEN DE BACTÉRIES FACULTATIVES

P.B. Altringer, D.M. Larsen, et K.R. Gardner

Centre de Recherche Salt Lake City,
Bureau des Mines, Département de l'intérieur des États-Unis
Salt Lake City, UT, É.-U.

RÉSUMÉ

Le Bureau des Mines du Département de l'intérieur des États-Unis mène actuellement des recherches en laboratoire sur l'extraction bactérienne du sélénium contenu dans les eaux usées. Une culture bactérienne mixte a permis d'extraire jusqu'à 96% du sélénium contenu dans des eaux de drainage agricoles dans un réacteur à petite échelle à écoulement idéal continu pendant une période de 1 an. L'extraction du sélénium a une relation réciproque avec les modifications engendrées dans les cultures bactériennes. Des données sont présentées concernant les relations complexes entre la réduction du sélénate et d'autres composés comme le nitrate, le phosphate et le sulfate. Des études sur les modes de respiration bactérienne, des résultats d'études de diffraction des rayons X et des prises de vue par microscope électronique à balayage du produit sont présentés pour clarifier davantage les mécanismes d'extraction du sélénium. Les résultats montrent aussi que les bactéries isolées dans les eaux de drainage agricoles peuvent servir avantageusement à extraire le sélénium contenu dans l'eau du bassin de déchets miniers.

INTRODUCTION

In 1983, the U.S. Fish and Wildlife Service determined that fish and waterfowl inhabiting the U.S. Department of the Interior's Kesterson National Wildlife Refuge in the San Joaquin Valley, CA, were being adversely affected by ingested selenium. Selenium is a necessary nutrient as a trace element, but in concentrations found at Kesterson, it causes malformation and death to wildlife. The Bureau of Mines began investigating the selenium contamination problem because the Kesterson National Wildlife Refuge is under the stewardship of the Department of the Interior (DOI). Since the problem at the Kesterson Reservoir was brought to light, selenium contamination has been identified at similar sites in five additional Western States. Selenium has recently been detected in precious metals tailings pond water and heap leachates as well as in other wastewaters resulting from a number of mineral processing operations (Hoffman, 1984). These results from Bureau research will have broad application.

A novel approach to solving this complex problem is the utilization of natural biological systems. Microorganisms are known to alter selenium oxyanions via several mechanisms including: (1) chelation (Brierley, 1982; Foster, 1983; Summers *et al.*, 1978; Trevors *et al.*, 1985), (2) volatilization (Brierley, 1982; Foster, 1983; Summers *et al.*, 1978; Trevors et al., 1985), and (3) precipitation of insoluble, elemental selenium (Brierley, 1982; Foster, 1983; Summers *et al.*, 1978; Trevors *et al.*, 1985). Reduction of selenium salts usually results in the accumulation of selenium products inside the bacterial cells (Doran, 1982; Silverberg *et al.*, 1976; Fraley *et al.*, 1987). Most frequently, reduced selenium is bound to amino acid complexes and is released when the cell dies (Gerrard *et al.*, 1974; Silverberg *et al.*, 1976; Brown *et al.*, 1980). However, this intracellular phenomenon is not the only reduction pathway. Extracellular precipitation can also occur as a result of (1) interaction between selenium salts and secreted enzymes or metabolic wastes such as H_2S (Doran *et al.*, 1977; Smith, 1959), and (2) reduction of selenium oxyanions which are utilized as terminal electron acceptors for bacterial respiration under both aerobic and anaerobic conditions (Hudman *et al.*, 1985).

Research at the Bureau's Salt Lake City Research Center has focused on bacterial reduction of soluble selenium to the elemental state in agricultural drainage waters (Larsen *et al.*, 1987; Larsen *et al.*, 1989) and in precious metals tailings pond water. The Bureau has identified selenium-reducing bacterial groups in samples from four agricultural drainage water reservoirs; these bacteria immobilize and concentrate selenium in pond sediments. No selenium-reducing bacteria were found in one tailings pond sample; thus, bacteria isolated from the agricultural waters were used to treat this sample. The research objective is to reduce the selenium in contaminated waters to non-toxic levels using natural biological processes.

DESCRIPTION OF SITES AND WATERS

Agricultural Drainage Water

The Bureau of Reclamation constructed the 1,280-acre Kesterson Reservoir and the 85-mile San Luis Drain through the San Joaquin Valley between 1968 and 1975. Most of the selenium in the Kesterson Reservoir came from subsurface, agricultural drainage water collected in under-ground tiles and carried via the Drain into the Kesterson Reservoir in Merced County, CA. The same situation with underground tiles collecting subsurface, agricultural drainage water exists at the Ouray National Wildlife Refuge and the Stewart Lake Waterfowl Management area in Utah, both of which are contaminated with selenium.

Laboratory-simulated San Luis Drain water and two bulk water samples received from the Westlands Water District (Kesterson water) in March and June of 1988 were used for most of the research discussed in this report. Water samples were also collected from the Ouray National Wildlife Refuge, the Stewart Lake Waterfowl Management area, and Ashley Creek in September of 1987 and 1988. Selenium was present as selenate in all samples. Analyses are shown in Table 1.

Precious Metals Tailings Pond Water

A sample of precious metals tailings pond water was obtained from an active silver mine. The tailings pond is contaminated with cyanide (as high as 400 ppm) and selenium (5 ppm), as well as arsenic (0.2 ppm), copper (4.5 ppm), iron (16 ppm), silver (3.2 ppm), and zinc (157 ppm). Most of the selenium (about 80%) appears to be present as metal-selenium-cyanide bonded complexes (Krishnan *et al.*, 1974), also known as selenocyanates ($SeCN^-$). The remaining selenium was concluded to be selenate based on results from selenate radiotracer studies.

BACTERIA IDENTIFICATION

Cultures from water samples from each of the selenium-contaminated wildlife refuges all contained a similar set of gram-negative, selenium-reducing bacteria. The active selenate reducers were isolated and identified using common bacterial identification procedures such as gram stains, growth on selective media, and standard biochemical tests. More rapid identification was facilitated by use of the Roche Diagnostic Systems. Fifteen bacterial species were isolated from the Kesterson area, the predominant family was *Pseudomonadaceae*, followed by *Enterobacteriaceae*. The most active selenium reducers used in laboratory experiments, either as isolates or mixed cultures, were (1) *Pseudomonas alcaligenes*, (2) *Pseudomonas fluorescens*, (3) *Pseudomonas aeruginosa*, (4) *Citrobacter freundii*, (5) *Enterobacter aerogenes*, (6) *Enterobacter agglomerans*, (7) *Enterobacter cloacae*, and (8) *Klebsiella oxytoca*.

SELENIUM ANALYSIS

Total selenium and selenite were determined at the Salt Lake City Research Center through hydride generation atomic absorption spectrophotometry (AAS) on a Perkin-Elmer[1] 603 AAS with a Varian VGA-76 hydride generator. Selenite [Se(IV)] was determined directly by standard hydride generation. Total selenium was then determined after oxidizing all selenium in the sample to Se(VI) in a potassium persulfate-nitric acid digestion followed by a reduction to Se(IV) with 6 M HCl. Selenate was calculated as the difference (Presser *et al.*, 1984).

Rapid, convenient, and accurate selenium assays were obtained in some bacteriological studies, when selenium was present strictly as selenate, by radioactive tracer analysis. Low levels of a radioactive isotope of selenate (^{75}Se) were mixed into the wastewaters. The amount of selenium removed from the water was determined by filtering and counting the difference in radioactivity between fixed volumes of feed and treated solutions. These results were later confirmed using hydride generation methods.

SELENIUM REMOVAL FROM AGRICULTURAL DRAINAGE WATER

Preliminary Studies

Earlier work showed that bacteria indigenous to Kesterson water reduced selenate to selenite and eventually to elemental selenium (Larsen *et al.*, 1987; Larsen *et al.*, 1989). Mixed cultures from both the *Enterobacteriaceae* and *Pseudomonadaceae* families worked effectively depending on the reactor design which determined the amount of air present. A mixed culture of *Pseudomonas sp.* reduced the selenium most rapidly under anoxic conditions.

Mechanisms of Selenium Removal

Bacteria remove metal ions from solution either through extra-cellular or intra-cellular biochemical reactions using six basic pathways: (1) adsorption on cell walls, (2) enzymatic reactions on cell walls, (3) external precipitation by biological agents such as H_2S, CO_2, O_2, etc., (4) methylation reactions forming gaseous products, (5) incorporation into cell walls and proteins, and (6) cellular traps where the metal ion is retained or expelled. The following studies revealed that several mechanisms may be involved during reduction and precipitation of selenium by *Pseudomonas sp.* bacteria.

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[1] Reference to specific products does not imply endorsement by the U.S. Bureau of Mines.

Respirometer studies were conducted at the Idaho National Engineering Laboratory with *Pseudomonas alcaligenes* bacteria. Four different samples were tested with three replicates and the results were averaged: (1) media + live *Pseudomonas alcaligenes* cells, (2) media + live *Pseudomonas alcaligenes* cells + selenium, (3) media + dead *Pseudomonas alcaligenes* cells + selenium, and (4) a control buffer solution. The respiration rates using 4 g/L glucose or 5 g/L peptone as the carbon source are depicted in Figure 1. The oxygen uptake using peptone was about 13 times that of glucose; thus, peptone was a more viable nutrient source. The controls in Figure 1 (the buffer sample and the dead cells plus selenium samples) did not consume oxygen. The oxygen uptake rates for the live cells with and without selenium were identical (Fig. 1). Therefore, selenate had no adverse effect on the respiration of oxygen by *Pseudomonas alcaligenes*, but whether *Pseudomas alcaligenes* respire selenium was not determined.

Next we looked for evidence of intracellular accumulation or adsorption on cell walls. Samples of *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes* were submitted for transmission and scanning electron microscopy and energy dispersive X-ray analysis at the Veterans Hospital in Salt Lake City, UT. The results of these experiments showed that selenium is accumulated in bacterial cells at levels well above the surrounding environment. The selenium appeared to be scattered throughout the cell with no localized dense precipitates as reported by Silverberg *et al.*, (1976). Distinct morphological differences were noted between cells which had been exposed to selenium and cells in media lacking selenium (Fig. 2). Horizontal cross sections of bacteria unexposed to selenate are shown in the top photomicrograph. They appeared to be normal. The unexposed cells exhibited few internal aberrations and no irregularities in membrane or cell wall structure. Dark, irregular areas are shown in several cells from both exposed and unexposed bacteria. These dark areas were not localized to one area of the cell, but appeared to be randomly dispersed throughout the cytoplasm. These dark areas were electron dense spots and indicated high concentrations of phospholipids, which are associated with cells just prior to or during division and indicated intensive nuclear activity such as rapid copying of genetic material and high-volume protein synthesis. The unexposed cells were 2.5 μm long and 0.5 μm wide, which was indicative of wild *Pseudomonas alcaligenes* bacteria. Cells which had been exposed to selenate are shown in the center (horizontal cross section) and bottom (vertical cross section) photomicrographs; the vertical cross section is at a much higher magnification. Several differences were apparent: (1) the average cell was much shorter (1.7 μm), (2) the cells were more compact and appeared almost ovoid in shape, (3) the dark electron-dense regions were almost circular and confined to the nuclear region of the cell, (4) smaller, electron dense particles formed a circle around the larger dark area, (5) the plasma membrane and cell wall were extremely rough and irregular and exhibited the characteristics of cellular components which have been subjected to stresses such as adverse pH or ionic disruption, and (6) fewer cells were undergoing division. Although exact statistical data was not collected, the observed changes were sufficiently consistent and unique as to attribute their effects to the selenium. The control bacteria lacked any of the fine-structure mutations of the bacteria which had been exposed to the selenate. The form of the selenium was not determined, nor was it determined whether the selenium is incorporated into protein or other organic material.

The mechanisms of bacterial selenate reduction are very complex and strongly influenced by other complexes in solution. Batch kinetic tests were conducted to investigate these interactions in a temperature-controlled, water-jacketed, 3-L vessel with a shaft-driven impeller. One week prior to the start of each test, a 125-mL mixed culture of *Pseudomonas sp.*, predominantly *Pseudomonas alcaligenes*, was prepared from a standard culture as the inoculant. Immediately before conducting each batch test, 2.1 L of simulated Kesterson water and the vessel were sterilized. The simulated water contained 610 ppb Se as selenate and 5 g/L peptone or 40 g/L cantaloupe pulp as nutrient sources; peptone and cantaloupe each removed <4% Se in control tests. The temperature was controlled at 25°C, the stirring rate at 350 rpm, and nitrogen and air were precisely metered through the system at 40 and 10 cc/min, respectively. These parameters, which were determined to be optimum in earlier research

(Larsen *et al.*, 1989), provided anoxic conditions with about 2 ppm O_2 in solution. Excess gases exited the reactor through a condenser and bacteriocidal trap.

Figure 3 shows the results from a kinetic test series where the concentrations of total selenium, selenate, selenite, nitrate, phosphate, and sulfate were analyzed as a function of reaction time. Results show simultaneous reduction of selenate, nitrate, selenite, and phosphate; sulfate remained constant at 5 g/L over the entire test. Respiration studies confirmed denitrification under anaerobic or low oxygen tension conditions independent of the presence of selenium. After 24 hr, approximately 84% of the selenate was converted to the elemental form at which point the selenate appeared to increase with no presence of selenite, the intermediate species. Either the reduced selenium was reoxidized to selenate or there is a Se(II) oxyanion species not determined in the analysis. These curves demonstrate that the reduction of selenate is a two- and possibly three-step reaction process. Selenate [Se(VI)], is converted to selenite [Se(IV)], then possibly to Se (II) which is precipitated from solution as elemental selenium. The bacterial cell count remained constant at around 2 by 10^9 cells/mL. Bacterial content was determined by a direct microscopic count of live cells with a Helber counting chamber (Simmons *et al.*, 1944). Under anoxic conditions, the bacteria may substitute selenium oxyanions, nitrates, and other anions for oxygen as the terminal electron acceptor in respiration; the reduction preference appears directly related to the standard electrochemical reduction potential of the appropriate half-cell reactions.

The precipitate from simulated San Luis Drain water was analyzed to contain the following, in percent: 0.42 Se, 2.8 S, 7 P, 23.3 Ca, and 64.3 Cl. X-ray diffraction patterns positively identified elemental selenium in the precipitate.

Continuous Testing in Plug-Flow Reactor

Research indicated that selenium reductions approaching 100% were possible using a continuous reactor of appropriate design. The desired characteristics of such a reactor included (1) high internal surface area for maximum retention of bacteria in the system, (2) a provision for renewal of the biomass, i.e., sloughing and removal of the dead biomass, and (3) the ability to control oxygen availability in the system. A passive system with few mechanical devices would be advantageous. Based on these concepts, a small plug-flow reactor (PFR) was designed and constructed to emulate a passive system in the San Luis Drain upon scaleup.

The PFR consisted of a 13.72-m length of 1.905-cm-ID Nalgene 8000 plastic tubing packed with ceramic, high-surface area, 0.635-cm Berl saddles. Sterile sampling ports were attached to the PFR at each 3.05-m interval. Samples were periodically collected at each sampling port and from the composite effluent after various days of operation. The feed tank was sparged with air to ensure an initial aerobic environment in the PFR.

The PFR was operated successfully over a 1-yr period, achieving 96% Se removal at startup with an average of 87, 86, and 76% Se removal at steady state for the three feed solutions (Fig. 4). When operating parameters were changed, such as altering the feed solution or changing the flow rate, an initial upset in selenium removal was observed, followed by resumption of steady-state removal.

Initially, the PFR was filled with sterile media and inoculated with a mixed *Pseudomonas sp.* culture. After 1 week cell density in the PFR was 2.5 by 10^8 cells/mL. Sterilized, simulated San Luis Drain water spiked with a radioactive isotope of selenate was then pumped through the PFR continuously. Simulated San Luis Drain water contained 610 ppb Se as selenate; 5.0 g/L peptone was added as the nutrient source, and the water was pumped through the PFR at 0.42 mL/min. Assuming plug flow through the system, a 3.05-m length of reactor yielded a 24-hr retention time; total retention time in the system was 4.5 days. Test results, shown in Figure 4, indicate high (>90%) selenium removal is possible in a continuous system.

The continuous PFR operated for the first 9 months using sterilized drainage water. The following drainage waters were tested: (1) simulated San Luis Drain water containing 610 ppb Se, (2) actual Westlands water containing 380 ppb Se, and (3) a second sample of Westlands water containing 150 ppb Se (Table 1). Over this time period, the feed rate was slowly increased from 0.4 to 1.2 mL/min. At the highest flow rate tested, the system established a steady-state selenium removal of 53% after 8 hr, 63% after 16 hr, 74% after 24 hr, and 78% after 32 hr of retention time.

Unsterilized Westlands water was pumped to the biological reactor at 1.2 mL/min during the last 3 months of operation. Following 24 days of continuous operation with unsterile feed, the reactor maintained steady-state selenium removal of 55% after 8 hr, 67% after 16 hr, 70% after 24 hr, and 72% after 32 hr of retention time. These results were extremely encouraging; they indicated that the bacterial culture in the reactor successfully competed with the wild non-selenium reducing strains and maintained the level of selenium removal. Characterization of bacteria types in the reactor indicated selenium-reducing bacteria were still predominant even though the bacterial population experienced dynamic fluctuations in genera and family over time. The dominant family in the PFR transformed from *Pseudomonas alcaligenes* to *Enterobacteriaceae* with the genera *Klebsiella*, *Enterobacter*, and *Citrobacter* being the most commonly encountered in these anoxic conditions. Periodic seeding of the reactor with *Pseudomonas sp.* initially increased the population of this group, but the *Pseudomonas* population dropped off drastically over a period of days and did not increase selenium removal. The significance of the test results was that selenium removal remained constant at about 88% even though operating parameters were changed and unsterilized feed with changing bacterial populations was used (Fig. 4). These results show great promise for implementing the process in the field.

SELENIUM REMOVAL FROM PRECIOUS METALS TAILINGS POND WATER

Batch Testing

Early microbial research, involving selenium removal from a precious metals tailings pond water, was unsuccessful due to the solution's high natural pH and high cyanide concentrations; the selenium in this water is probably present as a selenocyanate. Removing the cyanide using a chemical oxidation procedure produced a solution ideal for bacterial selenium reduction. The oxidation procedure entailed adding H_2O_2 to reduce cyanide levels from 250 ppm to <10 ppm followed by NaOCl to further reduce cyanide concentrations to <1 ppm. In the process, the selenocyanate complex was disassociated and converted to selenate as determined by results from radiotracer studies using selenate tracer. In addition, the solution pH decreased from a natural 10.5 to 8.5, which is ideal for *Pseudomonas sp.* bacterial selenium removal. Samples of the chemically-treated tailings water contained 1 ppm CN, 4 ppm Se, and traces of silver. Exploratory research demonstrated that *Pseudomonas sp.* bacteria reduced the selenium concentration in the low-cyanide solution. Bacterial cell growth was excellent at 2.5 to 4 by 10^9 cells/mL. These initial tests were conducted by placing 125 mL of the chemically-treated water in a 250-mL flask with a sterile lid. Selenate isotope tracer and 5 g/L peptone (as a nutrient source) were added to each sample. After sterilization, each sample was inoculated with 1 mL of *Pseudomonas sp.* bacteria and placed on the shaker table. Samples were withdrawn periodically, centrifuged, and filtered through a 0.2-μm filter to remove bacteria and precipitated material, and analyzed for selenium using radio-tracer techniques; analyses were later confirmed using hydride generation atomic absorption spectrophotometry. After 4 days of contact with bacteria, 72% of the selenium was removed from solution. Further contact resulted in some selenium going back into solution.

Results from another test showed that cyanide, at concentrations greater than 2 ppm, was detrimental to selenium removal. Subsequently, a series of batch tests was conducted using increasing amounts of cyanide. Results indicated that cell growth occurred in solutions containing up to 30 ppm CN, but selenium was extracted only in the sample with the least amount of cyanide (2.3 ppm). Therefore, cyanide, a potent electron transport inhibitor, does not hamper cell growth, but does interfere with selenium reduction.

Continuous Testing in Columns

A glass column was filled with 0.635-cm ceramic saddles; the measured bed volume was 255 mL. Sterile precious metals tailings pond water, to which 5 g/L peptone had been added, was circulated upflow through the column, and then the column was inoculated with *Pseudomonas alcaligenes* bacteria. After approximately 1 week of circulation, the recycle tubing was removed and fresh precious metals tailings pond water, containing 4 ppm Se and 2 ppm CN, was pumped upflow through the column on a single-pass basis at a rate of 0.84 mL/min (5.1-hr retention time). Half of the water contained 10 g/L peptone and was sterilized before being used; the other half of the water entering the column was non-sterile. The best extraction achieved after approximately 1 month of operation was 79%. After a brief shut down for repairs, the original column was placed back in operation for another month. Selenium reduction was maintained at a level between 53 and 67%.

In an effort to increase selenium removal, a new column was installed with a larger volume for increased retention time, a different bacterial substrate for increased surface area, and a continuous culturing chamber added for constant bacterial inoculation. The glass column dimensions were 4-cm ID and 39-cm high; the bed volume was 975 mL. The bacterial substrate was "Tuffy" scrub pads, and an air-lift culturing chamber was installed on-line to supply fresh bacteria to the column. The dimensions of the continuous culturing chamber were 2.2-cm ID and 20-cm high, producing a volume of 76 mL; the column was filled with 66 mL of sterilized solution, leaving 10 mL of air space. A glass expansion chamber was installed on top to trap any foam produced. Sterile precious metals tailings pond water spiked with 10 g/L peptone was pumped downflow through the continuous culturing chamber at a flow rate of 0.105 mL/min (10.5-hr retention time). The chamber was sparged with air for optimum bacterial growth. Two streams were pumped upflow through the glass column to continuously process contaminated water and provide fresh bacteria and nutrient: (1) unsterilized contaminated water at a flow rate of 0.42 mL/min, and (2) continuous culturing chamber effluent at 0.105 mL/min, for a total flow rate of 0.525 mL/min (31-hr retention time).

The bacteria in the continuous culturing chamber remained pure *Pseudomonas alcaligenes* with no detectable contamination after 7 weeks of operation. Chemical analysis by atomic absorption revealed that only 14% of the 3.26 ppm Se was removed in the glass column; silver was reduced from 0.3 to 0.1 ppm, while the concentrations of arsenic, copper, iron, and zinc remained unchanged. The culturing chamber, which was designed to provide fresh bacteria to the column, removed more selenium than the column; selenium was reduced from 3.8 to 1.1 ppm or 71% removal in 10.5 hr. Research will continue to pursue this innovative combination of chemical and biological treatment to remediate precious metals tailings pond waters and heap leachate solutions.

CONCLUSIONS

Bacteria will remove selenium from both agricultural drainage solutions and chemically-treated precious metals tailings pond solutions by precipitation. The process is at least a two-step reaction in which selenate is reduced to selenite then possibly to Se(II) and eventually to elemental selenium, which precipitates from solution. Studies revealed that several mechanisms may be involved during reduction and precipitation of selenium by *Pseudomas sp.* bacteria. Research is continuing at the Salt Lake City Research Center to scale up reactor design, determine the most cost-effective nutrient, implement optimal growth conditions, and further elucidate the complex mechanisms involved in the selenium reduction process. It is envisioned that this research will lead to the development of a cost-effective process to alleviate the wide-spread selenium contamination problem being encountered in mineral processing and other industries throughout the Nation.

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TABLE 1

Water analysis of various samples, ppm

Sample Type	Selenium	Sulfate	Nitrate	Phosphate	Chloride
San Luis Drain[1]	0.61	5,000	48.0	25.0	1,400.0
Westlands — March	.38	2,760	860	<0.1	1,160
Westlands — June	.15	3,580	120	<0.1	323
Ouray — 1988	.035	404	24.9	ND	89
Stewart Lake — 1988	.063	680	130	ND	44.8
Ashley Creek — 1988	.112	1,010	23.3	ND	26.8

ND Not determined.

[1] Simulated Kesterson water was prepared in the laboratory to approximate a typical San Luis Drain water analysis provided by the San Joaquin Valley Drainage Program. Inlets to the Drain have been plugged, and the farm sumps vary greatly in composition. Simulated Kesterson water is recommended for screening studies in order to standardize research being conducted around the country.

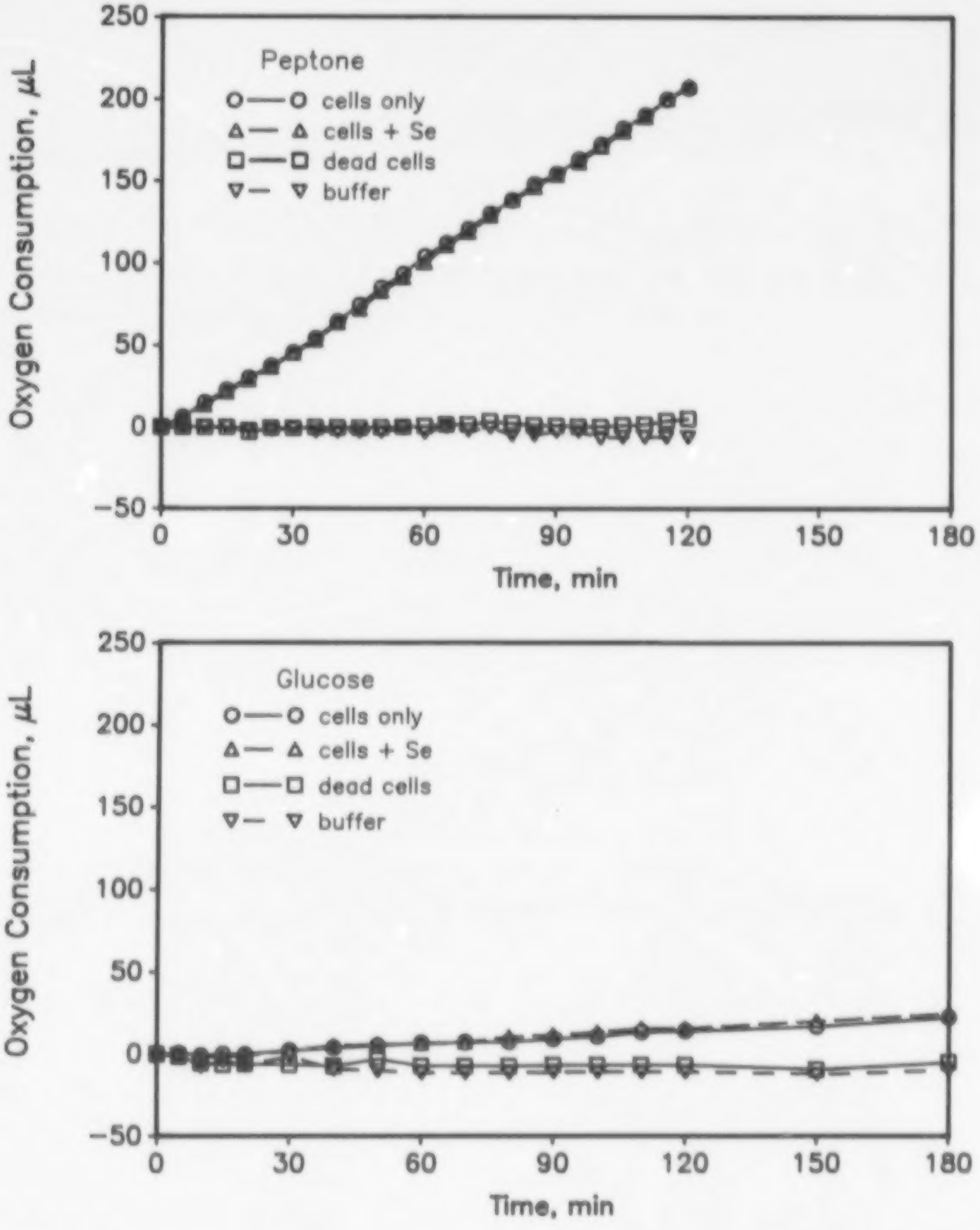

Fig. 1 *Pseudomonas alcaligenes* respiration rates using 4 g/L glucose or 5 g/L peptone as the carbon source.

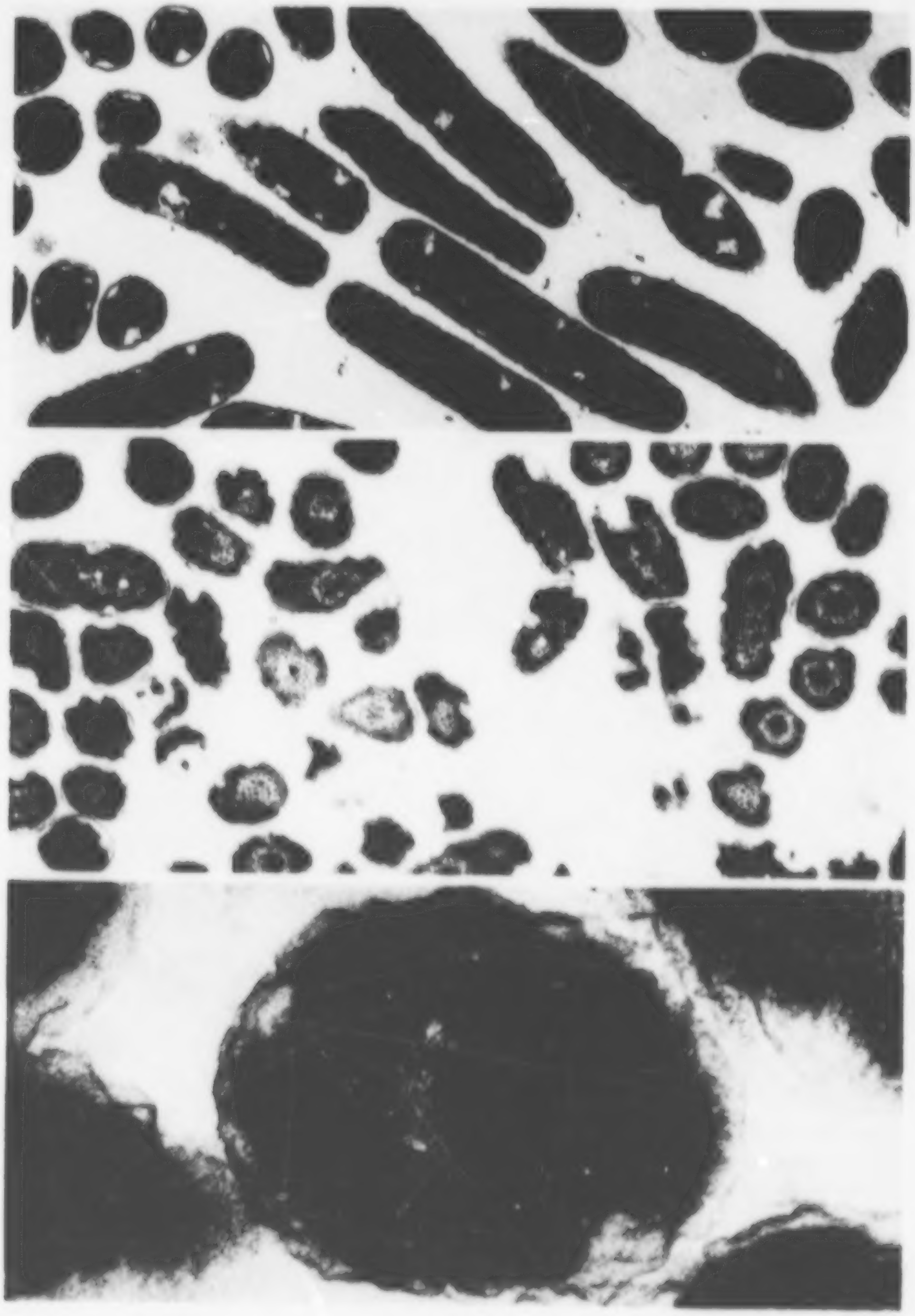

Fig. 2 Bacteria showing distinct morphological differences between cells which had not been exposed to selenium (top: horizontal cross section magnified 33,800X) and cells in media with selenium (center: horizontal cross section magnified 26,300X; bottom: vertical cross section magnified 182,000X).

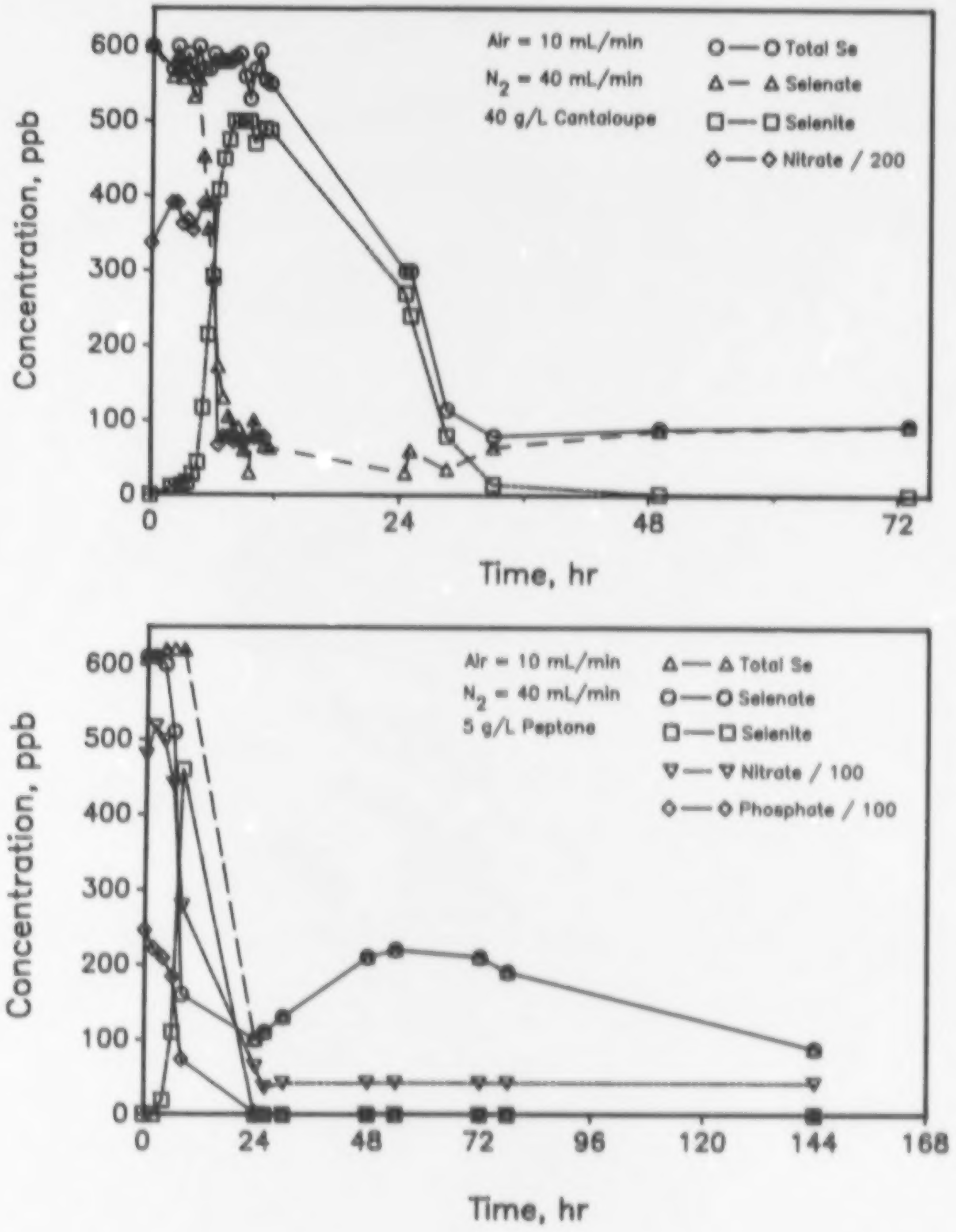

Fig. 3 Results from kinetic test series showing simultaneous reduction of total selenium, selenate, selenite, nitrate, and phosphate; sulfate remained constant at 5 g/L.

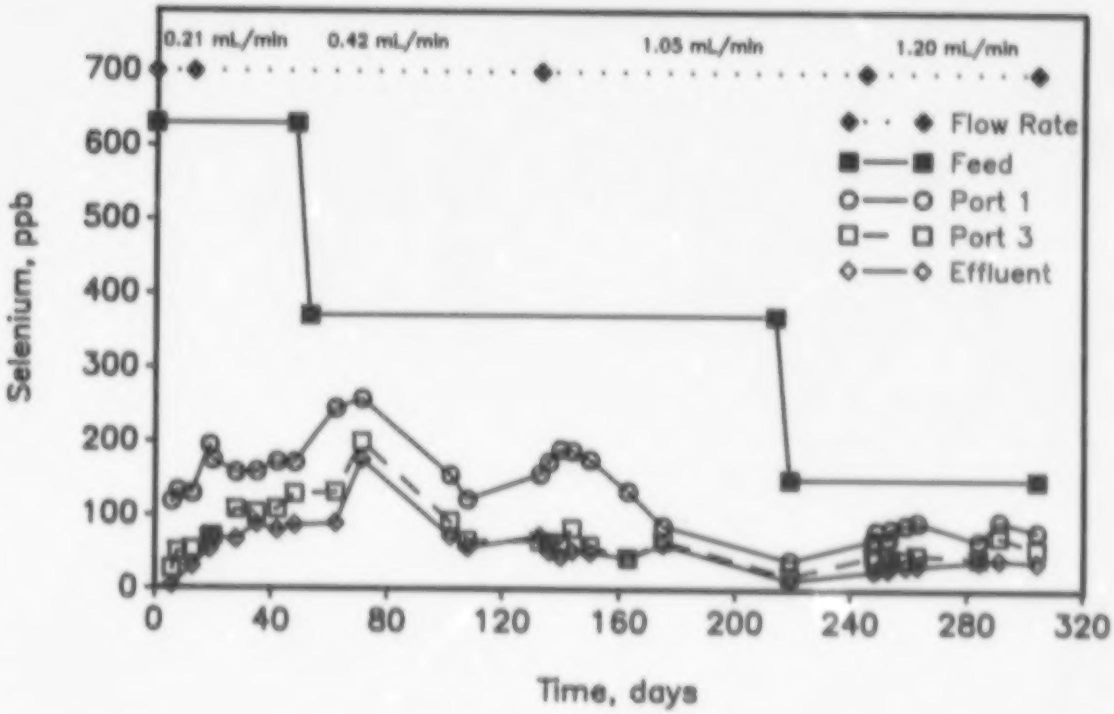

Fig. 4 Selenium reduction as a function of PFR operating time showing fluctuations when operating conditions were changed followed by resumption of steady-state selenium removal.

BIOLOGICAL POLISHING OF MINING WASTE WATERS: BIOACCUMULATION BY THE CHARACEAE

M. P. Smith and M. Kalin

Boojum Research Limited, Toronto, Canada

ABSTRACT

Nitella flexilis (an attached macrophytic algae) is evaluated for its use as a water treatment system for the removal of Ra^{226} and uranium from water leaving a uranium mine in northern Saskatchewan, Canada. The underwater meadow formed by the algae acts as a "filter" which, through its continuous growth and decay, relegates the contaminants from the water to the sediment. This natural treatment system, which is self-sustaining and maintenance free, is referred to as the Chara process. The underwater meadow has a density distribution of 100 g.m^{-2} to 1000 g.m^{-2}, in a water depth ranging from 0.6 to 1.9 m. The entire underwater meadow undergoes a complete growth turnover at least once per season. Concentrations of Ra^{226} and uranium in the biomass range from 10 to 30 Bq.g^{-1} and 660 mg.g^{-1} to 1290 mg.g^{-1}, respectively. Based on these findings, the Chara system was estimated to be capable of reducing the annual loading of Ra^{226} by 41 to 100% and the uranium loading by 1.5 to 3.2%. It was therefore concluded that the Chara process is a viable and effective ecological treatment process for Ra^{226}. Implementation was begun in 1989.

ADOUCISSEMENT BIOLOGIQUE DES EAUX USÉES D'EXPLOITATION MINIÈRE: BIOACCUMULATION PAR CHARA

M.P. Smith et M. Kalin

Boojum Research Ltée, Toronto (Ontario), Canada

RÉSUMÉ

On procède à l'évaluation de *Nitella flexilis* (une algue macrophyte fixée) en vue de son utilisation dans un système de traitement des eaux pour l'extraction du Ra^{226} et de l'uranium des effluents d'une mine d'uranium dans le nord de la Saskatchewan, Canada. Les algues forment une prairie sous-marine qui agit comme un filtre et qui, par leur croissance et leur décomposition continues, ramènent les contaminants présents dans l'eau à l'état de sédiment. Ce système de traitement naturel autonome et sans entretien est appelé procédé Chara. La distribution volumétrique de la prairie sous-marine est comprise entre 100 et 1000 $g.m^{-2}$, pour une profondeur d'eau comprise entre 0,6 et 1,9 mètre. Toute la prairie sous-marine est renouvelée complètement au moins un fois par saison. Les concentrations de Ra^{226} et d'uranium dans la biomasse sont respectivement comprises entre 10 et 30 $Bq.g^{-1}$ et 660 et 1290 $mg.g^{-1}$. À partir de ces résultats, on a évalué que le système Chara pouvait abaisser la charge annuelle de Ra^{226} de 41 à 100% et celle de l'uranium de 1,5 à 3,2%. On en a donc conclu que le procédé Chara constituait un procédé de traitement écologique efficace et viable du Ra^{226}. Sa mise en oeuvre a commencé en 1989.

INTRODUCTION

The treatment of waste water in mining can be a significant component of the operating budget. At the time of mine shut-down, treatment of waste water is frequently required, due to the continued production of unacceptable effluent from waste rock and tailings deposits that remain. Thus, the decommissioning of waste management areas in the ongoing attempt to ensure an acceptable and safe environment, often bears economic costs which are burdensome. Furthermore, with increasingly stringent environmental regulations, existing waste water treatment technology can be ineffective in achieving the required standards. Thus, the search for novel, cost-effective methods of achieving required environmental protection, both during mine operation and after shut down, is a necessary and ongoing process.

The Chara process has been developed for alkaline waters to assist mining companies to comply with these new environmental regulations. It is a biological polishing process which can both assist during operation to improve effluent quality and also provide a self-sustaining treatment system at the end of the mining operation. The potential for utilization of *Chara* (an attached, macrophytic algae) as a treatment system for alkaline waste streams was initially recognized by Brierley and Brierley (1981).

The uptake capacity of this family of algae is well known for the metals Zn, Cd, Co, Cr, Cu, Ni, and Pb, as well as for radionuclides such as Pb^{210}, Sr^{90}, Cs^{137} and Ru^{106} (Mudroch and Capobianco, 1978; Harding and Whitton, 1978; Dusauskene-Duz and Polikarpov, 1978; Marciulioniene *et al.*, 1976). The unique tolerance of the Characeae to alkaline waters with pH values up to 9.9 (Lucas *et al.*, 1983), with high suspended solids (Brierley and Brierley, 1981); their tolerance to elevated concentrations of Ni and Co (Strauss, 1986) and low nutrients (Hutchinson, 1975), suggested that this algal group could have excellent potential for utilization as a biological polishing agent (Kalin and Smith, 1985).

This paper reports on the biomass distribution and growth rates of the Characean underwater meadow within a uranium mining waste management area, as well as on the concentrations of Ra^{226} and uranium in the biomass. These parameters are used to determine the expected removal capacity of the Chara process for application in a drainage basin in northern Saskatchewan, Canada.

METHODS AND MATERIALS

Site description

The total area of the drainage basin is 10 km^2, and includes a mined-out open pit (formerly Lake 1) which is utilized as a tailings deposit, the uranium mill, the ore and waste-rock storage piles, Lake 2 and Lake 3 (Figure 1). Lake 2 is the first water body downstream of the pit (formerly Lake 1). It has a surface area of about 150,000 m_2 and an average depth of 1.5 m. A dam and overflow structure at the outlet of Lake 2 control the water level. From Lake 2, water flows through the Bog and a series of beaver dams before discharging into Lake 3, a lake very similar to Lake 2 (Vandergaast *et al.*, 1988).

The sources of Ra^{226} and uranium are waste rock piles located at the head of the drainage basin and Lake 1 sediments, which had reached the western portion of Lake 2 at the time the pit was developed (Figure 1).

In this drainage basin, it was noted that the concentration of Ra^{226} in the water decreases from 0.43 to 0.08 Bq.l·1, while uranium decreases from 0.60 to 0.18 $mg.l^{-1}$ between the outlet of Lake 2 and the inlet of Lake 3. The decrease occurs in a relatively small area of the Bog. Hydrological evaluations of the drainage basin indicated that concentration decreases in the Bog are much greater than can be accounted for by dilution from run-off. It was suggested that natural biological polishing is occurring within these locations. A dense population of *Nitella flexilis* (a Characean species) was identified in February 1988 in the main flow channels of the Bog.

Chara vulgaris and *Nitella flexilis* were found to be the major macrophytes in the lower part of the drainage basin in Lake 3, whereas Lake 2 was devoid of any macrophytic growth. Studies of the Bog and Lake 3 were therefore utilized to determine the required growth parameters for the application of the Chara process in Lake 2.

Biomass density and water depth

The *N. flexilis* populations' standing biomass and depth distribution in Lake 3 were determined in mid June, July and late August, 1988. Using an Eckman dredge, triplicate biomass samples were extracted from the lake bottom every 50 m along transects 200 m apart. The water depth was recorded at each sample location. Using a Li-Cor quantum sensor, a profile of light attenuation with depth was generated. Biomass samples were air dried (oven, 60°C), weighed, and standing biomass per square meter was calculated.

Turnover rates of biomass

Estimates of the growth and decay rates of *Nitella flexilis* were made at four locations; at the inflow and outflow of the Bog, and in the inflow and outflow of Lake 3. Plates, comprised of fifty 2.5 x 2.5 cm square acrylic rings temporarily held together with sucrose, were placed over the canopy of the population on June 15, July 11 and August 27. Upon dissolution of the sucrose, rings rest upon the *Nitella* canopy within 1 to 2 cm of the shoot apices, thereby labelling the uppermost portion of the population at that time. The amount of biomass, grown apically and decayed from the base, was determined using measurements of the ring positions relative to the sediment surface and plant apices at later dates. By successive placement of differently coloured rings and re-measurement of the ring positions at a later date, distances of apical growth and basal decay could be estimated based on the vertical movement of the rings in the underwater meadow. Distances of growth and decay were then translated into cumulative biomass estimates.

Growth of transplanted biomass

N. flexilis biomass collected from Lake 3 was spread inside plastic mesh 30 x 50 cm racks and weighted to the sediment surface at eight locations in Lake 2, at depths ranging from 0.4 m to 1.38 m. All shoots emerging from the racks were deemed to be new biomass. A rack from each of the areas was harvested at 30, 53, 79 and 107 days after transplant.

Radionuclide uptake

Biomass from inflows and outflows of the Bog and Lake 3 populations and emergent biomass from transplant racks was collected and washed free of particulates in lake water. The biomass was dried, ground and then submitted for wet digestion and analysis for Ra^{226} and uranium (Saskatchewan Research Council, Canada).

RESULTS AND DISCUSSION

Biomass distribution

An extensive population of *N. flexilis* covers 18 of 22 hectares of Lake 3's surface area, where water depth is between 0.6 and 1.9 metres. Between these depths, the standing biomass ranges from minor amounts to over 1000 $g.m^{-2}$, an average of approximately 100 $g.m^{-2}$ ($n=78$). This is within a similar range for standing biomass values reported to attain 1 m in height and levels as high as 1000 $g.m^{-2}$ in natural temperate populations (dry weight, mineralization free) (Andrews *et al.*, 1984; Forsberg, 1964; Westlake, 1965). Throughout the growing season, *N. flexilis* was never found below 1.9 m, the depth at which light was attenuated (in both Lake 2 and Lake 3) to 5% of the incident radiation. As the quantity of light diminishes with depth, the spectral quality of light which reaches the vegetation canopy also changes, affecting both the photosynthetic rates and the photomorphogenic switch mechanisms (Kirk, 1977). Thus, these factors determine the depth distribution and the standing biomass in Lake 3.

Biomass turnover

Data from the four zones with ring positions indicated that over 98 days of growing season, the cumulative growth of biomass ranged from 200 to 300 $g.m^{-2}$, and the cumulative decay ranged from 150 to 400 $g.m^{-2}$. These values suggest that the population is replaced at least once in the growing season, i.e. 100% turnover per season. A turnover rate of at least one time per year is also in agreement with both Rich *et al.* (1971) for a Michigan Characean population, and with observations of natural populations in abandoned gold tailings in northern Ontario, Canada (Kalin and Smith; 1987, 1988).

Growth of transplanted biomass and radionuclide uptake

Nitella transplanted from Lake 3 grew successfully in Lake 2, reaching up to 555 $g.m^{-2}$ (Figure 2). This was in excess of all biomass production values determined from the Bog and Lake 3. These results are in accordance with earlier work where vegetative portions of the plants were manually transplanted over completely inorganic substrates (gold tailings) (Kalin and Smith, 1988). From an initial coverage of 10% of newly unearthed tailings, the transplanted populations attained 80% coverage over the course of one growing season.

Emergent biomass from the transplant racks in Lake 2 had the highest Ra^{226} content, 13 to 30 $Bq.g^{-1}$ (Table 1). In the Bog, the radium concentrations were somewhat lower, averaging 7.3 to 8.2 $Bq.g^{-1}$. In Lake 3, average radium concentrations had decreased to between 1.7 and 2.7 $Bq.g^{-1}$.

CONCLUSIONS

An illustration of the Chara process is shown schematically in Figure 3. The underwater Characean meadow's high surface area represents the "filtration zone" of Characean populations. Biomass of 100 $g.m^{-2}$ translates into approximately 10 m^2 plant surface area per m^2 of population (Smith, 1987). Standing biomass increases with apical growth and is balanced, in the long term, by death and decay of the basal portions of the populations.

As apical growth of the population occurs, it will provide new surface area for filtration. Simultaneously, lower parts lie on anaerobic sediments and senesce (basal decay). As the influent is released into the underwater meadow, contaminants (both in dissolved and algal particulate forms) are loaded onto the filtration zone. As the distance from the influent discharge point increases (distance axis), the water is cleansed, until, at point d_x and time T_1, the last of the filterable contaminant is removed.

T_o (not shown) is the time of implementation of the Chara process. Sediments deposited prior to implementation (brick shading; e.g. at the west end of Lake 2) are subsequently covered by sediments laid down by the Chara process (light and dark shading). At any time after implementation (time axis), say time T_1, and at a given location (d_o) - the "front" on the schematic — the sediments deposited by the Chara process will have attained a certain quantity of removed contaminants (light shading above brick shading). The concentration of contaminant in the sediment layer diminishes with distance downstream to the location at which contaminant fixation is no longer occurring (d_x; Lake 2 outflow). With the passage of time towards T_2, the thickness of the sediment layer will increase (dark shading).

From the ecological characteristics of the underwater meadow in Lake 3, it is indicated that, upon colonization of the depth range available for growth in Lake 2, a *N. flexilis* population would achieve a standing biomass averaging 100 $g.m^{-2}$, turning over at least once per year. As 17 hectare of Lake 2 is between 0.6 and 1.9 m deep, biomass production will therefore be approximately 1.7×10^7 g, or 17 metric tonnes per year. As emergent *N. flexilis* biomass transplanted to Lake 2 contained 10 to 30 Bq $Ra^{226}.g^{-1}$, bioaccumulation per year could range from 1.7×10^8 to 5.1×10^8 Bq. The annual Ra^{226}

loading to Lake 2 from the waste rock piles and the sediments is about 4.1×10^8 Bq. Uranium bioaccumulation ranged from 0.66 to 1.29 $mg.g^{-1}$. Uranium bioaccumulation is therefore expected to range from 1.1×10^7 to 2.2×10^7 $mg.yr^{-1}$. The annual loading of uranium is 6.9×10^8 $mg.yr^{-1}$ from the same sources.

These estimates suggest that, upon implementation, the Chara process could conceivably remove most of the Ra^{226} from solution but only a fraction of the uranium. The *N. flexilis* productivity value of 100 $g.m^{-2}.yr^{-1}$ is conservative, since the transplants reached 550 $g.m^{-2}$ in 107 days in Lake 2. At present, no estimates exist to assess the filtration capacity of the Chara process.

All samples of *Nitella* biomass were washed prior to drying; most of any additional Ra^{226} and uranium removed by periphytic algal communities colonizing the *N. flexilis* population would have been lost during this procedure. As well, a reduction in contaminant loading to the water will take place, due to a reduction in contaminant flux upon coverage of the sediments with the *Nitella* population and the formation of new sediment, as indicated in Figure 3.

The results of the feasibility study indicate that implementation of the Chara process at the northern Saskatchewan site provides a viable solution to reducing the elevated concentration of Ra^{226} in the water leaving the drainage basin. The implementation of the Chara process in Lake 2 commenced in the spring of 1989.

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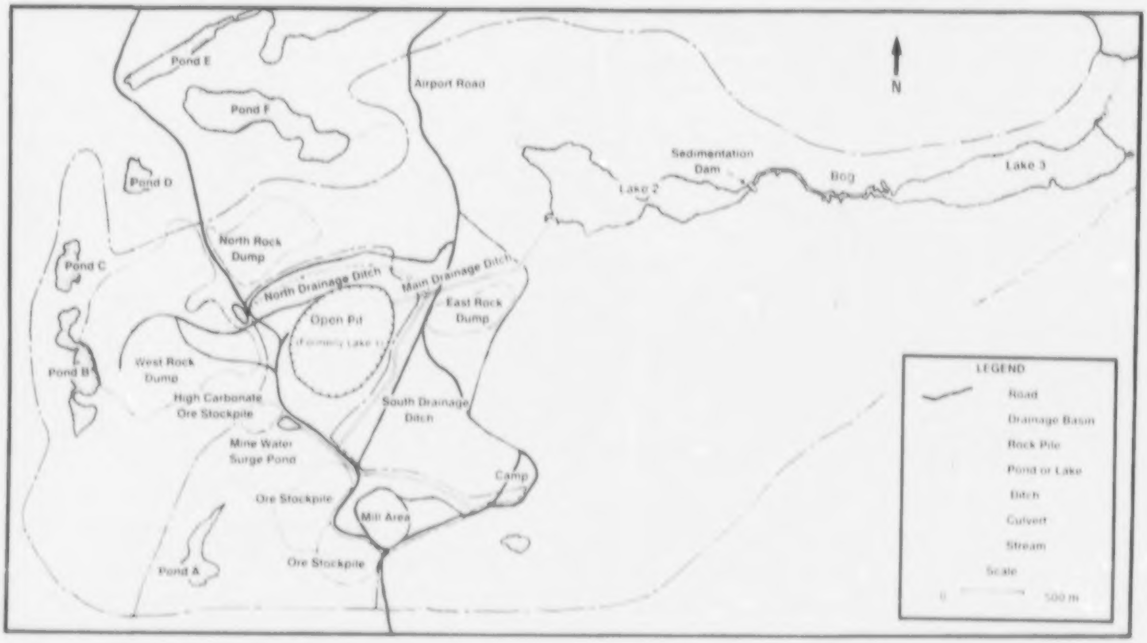
N
Pond E
Pond F
Airport Road
Pond D
Sedimentation Dam
Lake 2
Bog
Lake 3
Pond C
North Rock Dump
North Drainage Ditch
Main Drainage Ditch
East Rock Dump
Open Pit
(formerly Lake 1)
Pond B
West Rock Dump
High Carbonate Ore Stockpile
South Drainage Ditch
Mine Water Surge Pond
Camp
Ore Stockpile
Mill Area
Pond A
Ore Stockpile
LEGEND
Road
Drainage Basin
Rock Pile
Pond or Lake
Ditch
Culvert
Stream
Scale
0
500 m

Fig. 1 Overview of the drainage basin located in northern Saskatchewan, including the mill area, the mined-out pit (formerly Lake 1), the waste rock dumps, Lake 2, the Bog and Lake 3.

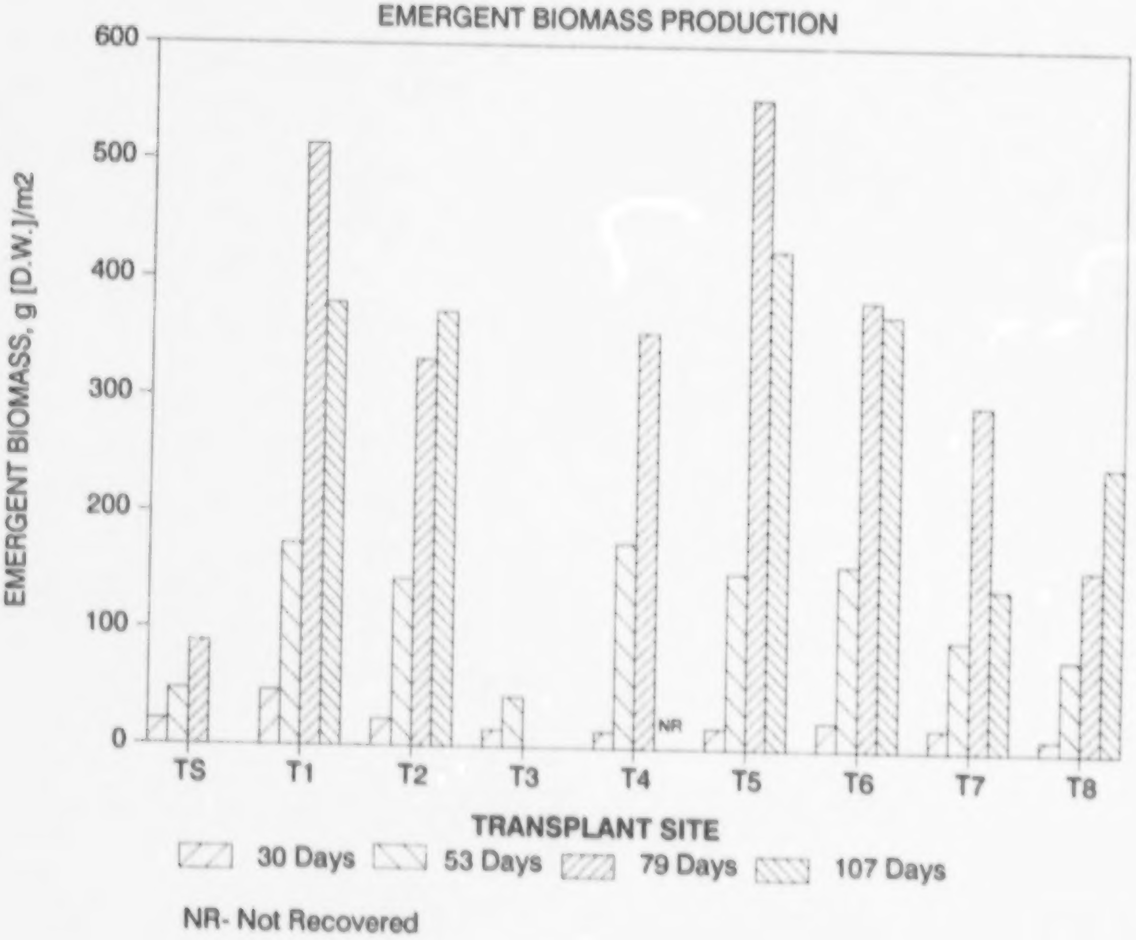

Fig. 2 Emergent (net) biomass production (g.m^{-2}, dry) by *Nitella flexilis* biomass transplanted June 5, 1988 in Lake 2.

LOCATION	SAMPLE HISTORY	1988 SAMPLE DATE	[RADIUM 226] Bg / g (D.W.) X	S.D.	N	[URANIUM] ug / g (D.W.) X	S.D.	N
Lake 2 Transplants								
Above Narrows	30 days	6 / 7	12.6	6.3	4	660	3294	4
	53 days	29 / 7	21.8	3.1	5	1282	423	5
	79 days	24 / 8	20.3	2.9	4	1296	635	4
	107 days	21 /9	19.5	0.9	4	826	419	4
Below Narrows	30 days	6 / 7	30.0	0	1	660	0	1
	53 days	29 / 7	21.0	1	2	1284	840	2
	79 days	24 / 8	15.0	0	1	1296	0	1
	107 days	21 / 9	17.5	1.5	2	926	20	2
Bog Standing Biomass		25 / 2	8.2	7.3	5	2528	717	4
		1 / 8	7.25	1.9	4	1566	1016	4
Lake 3 Inflow Standing Biomass		25 / 2	2.7	2.1	2	994	206	2
		1 / 8	1.9		1	1290		1
Lake 3 Outflow Standing Biomass		25 / 2	1.67		1	1400		1
		1 / 8	2.25	0.3	2	1238	612	2

Table 1 Concentrations of Ra^{226} and uranium in *Nitella flexilis* biomass.

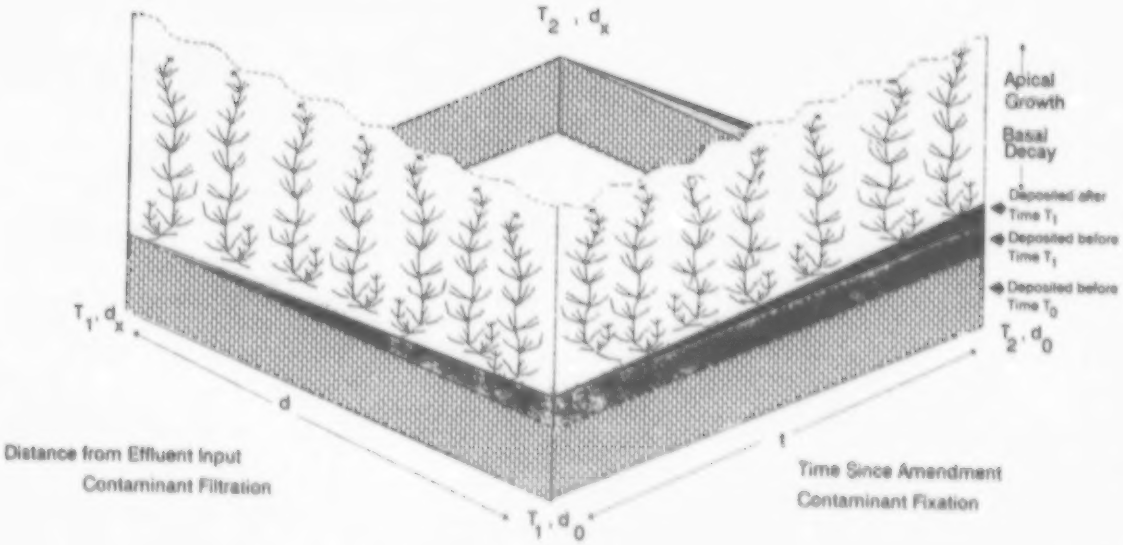
T_2 , d_x
Apical Growth
Basal Decay
Deposited after Time T_1
Deposited before Time T_1
Deposited before Time T_0
T_1, d_x
T_2, d_0
d
t
Distance from Effluent Input
Contaminant Filtration
Time Since Amendment
Contaminant Fixation
T_1, d_0

Fig. 3 Configuration of the Chara process.

URANIUM ACCUMULATION AND RECOVERY BY IMMOBILIZED CITROBACTER SP. IN A CONTINUOUS FLOW-THROUGH BIOREACTOR: BIOREACTOR MODELLING AND CHLORIDE-INTERFERENCE EFFECTS

L. E. Macaskie

Microbiology Unit, Department of Biochemistry
University of Oxford, South Parks Road, Oxford OX1 3QU

ABSTRACT

These studies demonstrate the use of a mathematical model to describe the removal of uranium from challenge flows presented to an immobilized cell bioreactor. Using this approach anion-interfering effects can be quantified in terms of bioreactor performance.

ACCUMULATION ET RÉCUPÉRATION D'URANIUM PAR *CITROBACTER* SP. IMMOBILISÉE DANS UN BIORÉACTEUR À FLUX CONTINU: MODÉLISATION DU BIORÉACTEUR ET EFFETS PERTURBATEURS DES CHLORURES

L. E. Macaskie

Unité de Microbiologie, Département de Biochimie
Université de Oxford, Oxford U.K.

RÉSUMÉ

Les présentes études démontrent l'utilisation d'un modèle mathématique en vue de décrire l'extraction de l'uranium dans des écoulements à l'intérieur d'un bioréacteur à cellules immobilisées. Par cette approche, il est possible de quantifier les effets de l'interférence anionique en termes de rendement du bioréacteur.

INTRODUCTION

Immobilized biofilms of a *Citrobacter* sp. accumulate uranium continuously via the activity of a heavy metal-resistant cell surface located phosphatase which liberates HPO_4^{2-} from glycerol 2-phosphate with stoichiometric precipitation of heavy metals as cell-bound $MHPO_4$. Phosphatase activity obeys Michaelis-Menten kinetics and an integrated form of the Michaelis-Menten equation relates phosphatase activity, substrate cleavage efficiency and flow rate in immobilized whole cells in flow-through columnar bioreactors. (1) Using two biomass supports co-colonized in a single growth run, a kinetic model was developed to assess bioreactor performance. (2) Proof of this is given in the present communication and an extension of the model is employed to quantify the effects of interfering anions on uranium removal from challenge flows.

BIOREACTOR MODELLING

By integrating the Michaelis-Menten equation to describe enzyme activity in a flow through (plug flow) reactor, the following is obtained:

$$E_oK_3 = F[S_oX + K_m.\ln(1/(1-X))] \tag{i}$$

F = flow rate. K_m,K_3 = Intrinsic kinetic contents.

E_o = total enzyme available. S_o = Input substrate concentration.

X = conversion factor, = (So-S)/So.

S = substrate concentration at time t, where t = 1/F.

But UO_2^{2+} removed = HPO_4^{2-} liberated = substrate (glycerol 2-phosphate) cleaved. Therefore, X can be redesignated as U removal from the flow, either as removal efficiency (O-100%) or as fractional efficiency (O-1), with Input $[UO_2^{2+}]$ expressed as U_o.

The actual value for Km does not affect the calculation, (2) for convenience Km here is taken as 1.

Given two values for E_o (at F = constant):

$$\frac{E_{o1}\,K3}{E_{o2}\,K_3} = \frac{F\,[U_oX_1 + \mathrm{ln.}(1/(1-X_1))]}{F\,[U_oX_2 + \mathrm{ln.}(1/(1-X_2))]} \tag{ii}$$

Or, more simply:

$$E_{o1} = \frac{E_{o2}\,[U_oX_1 + \mathrm{ln.}(1/(1-X_1))]}{U_oX_2 + \mathrm{ln.}(1/(1-X_2))} \tag{iii}$$

E_o represents the product of activity due to three subparameters:

$$E_o = \begin{bmatrix}\text{phosphatase}\\ \text{titre/cell, pt}\end{bmatrix} \begin{bmatrix}\text{bioreactor colonized}\\ \text{area, ba}\end{bmatrix} \begin{bmatrix}\text{reaction}\\ \text{temp, rt}\end{bmatrix}$$

(This assumes that the biofilm available depth is constant; this has been shown previously). Thus:

$$[pt_1][ba_1][rt_1] = \frac{[pt_2][ba_2][rt_2][U_oX_1 + \mathrm{ln.}(1/(1-X_1))]}{U_oX_2 + \mathrm{ln.}(1/(1-X_2))} \tag{iv}$$

In other words, by splitting the Eo parameter by introducing known changes in phosphatase titre, surface area and reaction temperature values for X can be calculated for known E_o and compared with values for X obtained directly by experiment.

EXPERIMENTAL SYSTEM

1. *Pre-growth and biofilm formation Growth conditions:* Carbon (glycerol)-limiting continuous culture

   Phosphorus source: Glycerol 2-phosphate

   Tris-buffered inorganic salts medium, pH 7.1, 30°C

   maintained at: 0.25 μ^{max} (D = 0.13/h); 6 days

   Growth Fermenter: Tower (air-lift) fermenter with support material

   Immobilized biofilm Polyurethane reticulated foam supports: (Recticel Ltd)

   Porous glass raschig rings (Schott Glass Ltd)

2. *Metal accumulation Bioreactors:*

   Biofilm-colonized material packed in 140 ml flow-through columns

   Challenge solution: | 5 mM glycerol 2-phosphate
   pH 6.9 — | 1 mM uranyl nitrate
   | 2 mM citrate buffer

   Flow rate: As detailed in individual experiments

   Determination of bioreactor efficiency:

   By assay of U input and output using standard colorimetric assay

   Expressed as either X (see bioreactor modelling) or $FA^{1/2}$ (that flow rate giving 50% efficiency

3. *Chloride inhibition:* NaCl incorporated to required final [Cl] as indicated

RESULTS

Flow rate-activity relationships for strains N14 and dc5c (phosphatase activities as shown in Table 1) are shown in fig. 1 where the $FA_{1/2}$ valve is defined as that flow rate giving 50% removal of uranium from the flow. The data are replotted as shown in Fig. 2: for convenience only linear regression lines are shown (data points omitted) with the high correlation coefficient justifying this linearization. The F threshold criterion (that flow rate at which activity starts to be lost) gave identical results in calculation to $FA_{1/2}$. Fig. 2 shows also the increase in activity ($FA_{1/2}$) afforded by increasing the temperature to 30°C. By use of two supports (areas as shown in Table 2) the known value for E_o can be manipulated by known variations in each of the E_o subparameters, i.e., phosphatase activity, surface area and reaction temperature.

The effect of chloride as an interfering anion was quantified using the mathematical model. Flow rate activity relationships were obtained using bioreactors initially chloride-free and then supplemented with chloride to obtain a new value for X at f = constant. Using the determined values for X it was possible to calculate an apparent value for E_o at each chloride concentration tested. In these experiments the supports employed were TR20 reticulated foam as before, and commercially available "pumice sponge". In the absence of added chloride, the ration of $FA_{1/2}$ values for the two supports was 4.57 (Table 3). The effect of chloride is shown in Fig. 4; the effect on E_o was related to the log of the chloride concentration, with the foam-immobilized cells more tolerant to the anion. At E_o = 30% of the controls, the chloride concentration giving this effect was calculated (Fig. 4). The ration for these values was identical to that obtained in the absence of chloride for the two supports (Table 3) and justifies the use of this model to quantify anion interference.

DISCUSSION

The techniques described provide a working system to assess quantitatively the effects of anions incorporated into metal perfusates presented to the biofilm bioreactors. This type of approach will enable predictive modelling to be employed to tackle the problem of highly complex wastes (e.g., those produced by nuclear fuel reprocessing) most effectively. The latter comprise a mixture of cationic and anionic species, with some anions present to great excess (3). Anion interference is generally by the formation of metal-anion complexes, reducing metal availability. This is very difficult to model in mixed cationligand solutions. the elegance of the present approach is that it is the net result of these interactions (and also any direct effects on the enzyme) that produce the shift in bioreactor activity. It would be thus easy to predict alterations to the flow rate, bioreactor support surface area and enzyme loading in order to compensate for the loss in activity due to co-pollutant interference. This provides an important first step in the application of the bioprocess to actual waste situations.

ACKNOWLEDGEMENTS

The author wishes to acknowledge, with thanks, the financial support of BTP Ltd. towards the research programme, in association with Pembroke College, Oxford. The work was carried out by Miss R.M. Empson and Mr. M.R. Tolley as part of their undergraduate research projects (FHS Biochemistry, University of Oxford).

Bioreactor Modelling was performed using the flow rate-activity relationship for several values of E_o using the split E_o parameter as shown in fig. 3. At F = constant (here 10 ml/min) values for X were determined using the experimental data. The least effective combination (strain dc5c, 20°C) was given the value E_o = 1 (by assigning a value of 1 to each of the Eo subparameters); this corresponded to X = 0.13 (Fig. 3). Values for X were calculated by substitution into equation (iv) and were in good agreement with those obtained directly by experiment. (Table 2)

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Table 1

Bioreactor manipulations employed to obtain changes in E_o

Phosphatase specific activity	Strain dc5c	Strain N14	Theoretical Increase in E_o
(nmoles p-nitrophenol/ min/mg protein)	1404	3743	2.67-fold
Surface area of support (total area/bioreactor)	Raschig rings 0.056m^2	Reticulated foam 0.126m^2	2.25-fold
Bioreactor running temperature	20°C	30°C	2.00-fold

Table 2

Comparison of values for X obtained by experiment with those obtained by calculation using the mathematical model

	Value for E_o	Experimental conditions	X actual (Fig. 2)	X calculated (eqn. iv)	Difference (%)
$1.00E_o$		Strain dc5c, raschig rings, 20°C	0.13	–	–
$2.67E_o$	Phosphatase titre ↓	Strain N14, raschig rings, 20°C	0.29	0.32	9.4%
$6.00E_o$	Biomass support ↓	Strain N14, reticulated foam, 20°C	0.53	0.63	16%
$12.00E_o$	Operational temperature ↓	Strain N14, reticulated foam, 30°C	0.70	0.90	22%

Table 3

Application of the model to describe chloride inhibition of the bioreactor

	Reticulated foam immobilized cells (A)	Pumice sponge-immobilized cells (B)	Ratio A/B
Bioreactor activity chloride unsupplemented ($FA^{1/2}$)	113.35 ml/min	2.92 ml/min	4.57
Chloride concentration giving 30% of control E_o	135 mM	29 mM	4.65

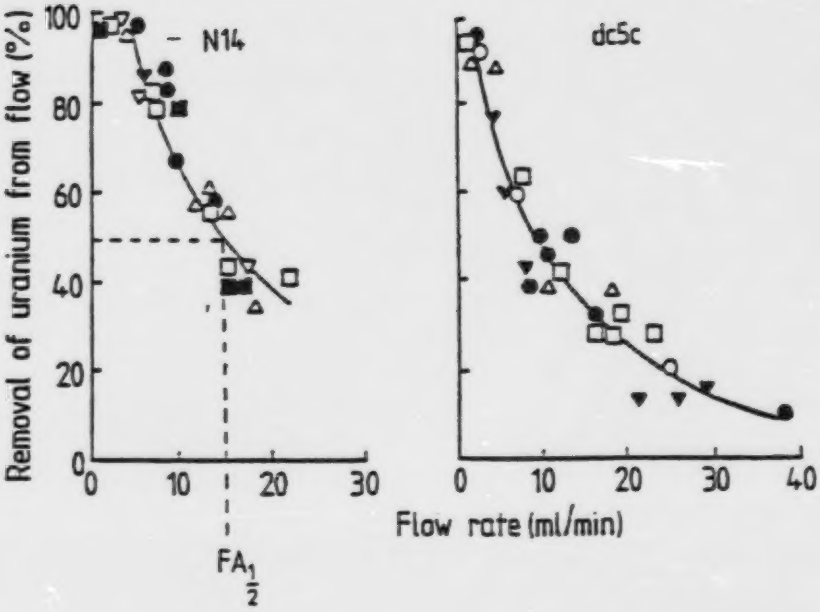
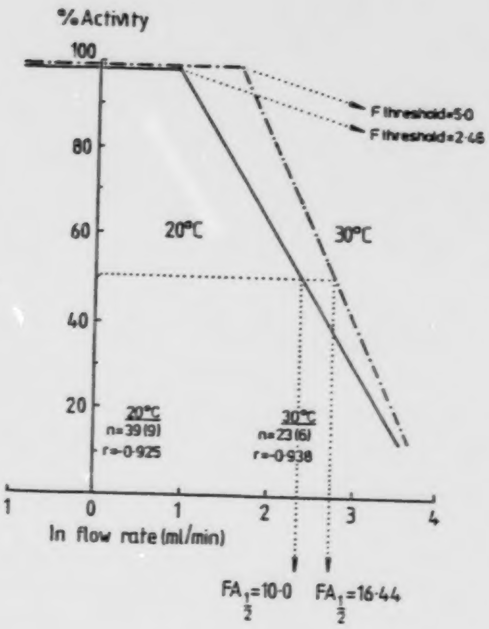


Fig. 1 Flow rate-activity relationships for strains N14 and dc5c immobilized on reticulated foam and challenged with U-solution at 30°C. Each symbol represents a separate experiment.

STRAIN N14, reticulated foam



STRAIN dc5c, reticulated foam

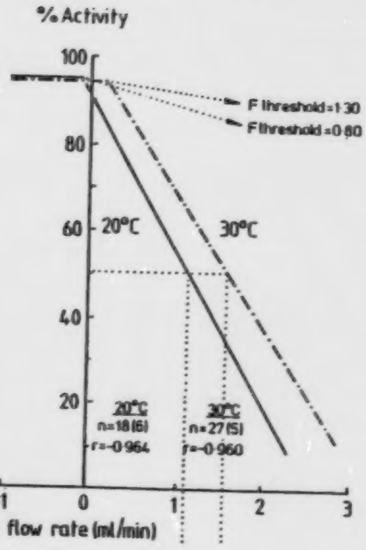


Fig. 2 Experimental data are omitted for clarity. The plots show linear regression lines for data as in Fig. 1 replotted as X (percentage efficiency of uranium removed) versus ln of the flow rate, F. r = correlation coefficient for n data points; pooled data from several experiments.

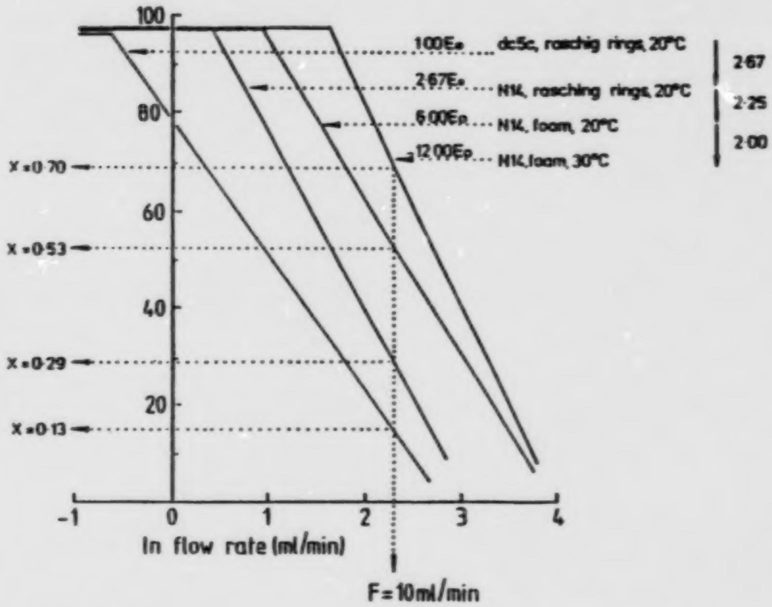


Fig. 3 Use of different experimental situations to obtain known values for E_o subparameters, and determination of X from the flow rate-activity relationships

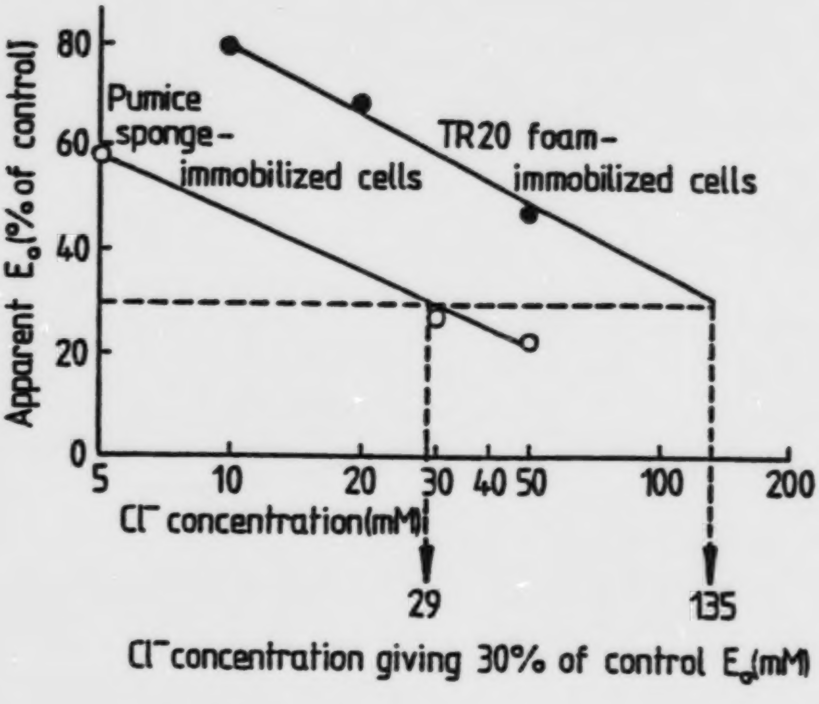


Fig. 4 The effect of chloride on E_o : E_o was calculated by substitution into eqn. (iii) using values for X determined by experiment under Cl-free and Cl-stressed conditions. It should be emphasized that the change in E_o is apparent and is here the net result of the effect of chloride on the phosphatase enzyme and interference with the availability of UO_2^{2+} for precipitation by metal-ligand complex formation.

FOSSIL FUEL

BIOTECHNICAL METHODS OF SELECTIVE PLUGGING TO ENHANCE OIL RECOVERY

F.M. Cusack, H.M. Lappin-Scott,

and

J. William Costerton

Department of Biological Sciences The University of Calgary
Calgary, Alberta Canada T2N 1N4

ABSTRACT

The permeability of subterranean oil-bearing formations is selectively reduced by the injection of microorganisms into the formation. The micro-organisms produce biofilms that selectively seal off high permeability zones within the reservoir so that a subsequent waterflood may enter the less permeable oil-bearing zone. Microbial experiments to study enhanced oil recovery have generally been conducted using vegetative microorganisms and one-dimensional laboratory models. We have developed a procedure utilizing microorganisms called ultramicrobacteria (UMB) or starved bacteria (.2 – .3 μm) that have the ability to penetrate deep into model rock cores and respond to nutrient stimulation *in situ*. Vegetative cells or full-size cells tend to form "skin plugs" at or near the well bore. to simulate reservoir conditions, UMB experiments were conducted in a radial sandpack, 48 cm in diameter x 28 cm in height, designed by the Alberta Research Council. Our experiments have shown: 1) uniform penetration and resuscitation upon nutrient stimulation of UMB, and 2) resuscitated UMB preferentially plug the high permeability zone by virtue of their growth properties and extracellular polysaccharide (polymer) production.

MÉTHODES BIOTECHNIQUES DE COLMATAGE SÉLECTIF EN VUE D'AMÉLIORER LA RÉCUPÉRATION DU PÉTROLE

F.M. Cusack, H.M. Lappin-Scott,

et

J. W. Costerton

Département de Sciences Biologiques,
Université de Calgary, Calgary (Alberta), Canada

RÉSUMÉ

La perméabilité des formations pétrolifères souterraines est réduite de façon sélective par injection de micro-organismes dans la formation. Les micro-organismes produisent des biofilms qui colmatent de façon sélective les zones de grande perméabilité à l'intérieur du réservoir pour que l'eau admise ultérieurement s'infiltre dans la zone pétrolifère moins perméable. Afin d'étudier l'amélioration de la récupération du pétrole, des expériences microbiennes ont généralement été menées au moyen de modèles de laboratoire unidimensionnels et de micro-organismes végétales. Nous avons mis au point une méthode utilisant des micro-organismes appelés ultramicrobactéries (UMB) ou bactéries carencées du point de vue nutritionnel (0.2 à 0.3 μm) qui ont une aptitude à pénétrer en profondeur dans le noyau rocheux du modèle et à réagir au stimulus d'éléments nutritifs *in situ*. Des cellules végétales ou des cellules grandeur nature ont tendance à former des "membranes d'obturation" à l'orifice du puits ou à proximité de celui-ci. Pour simuler les conditions du réservoir, on a mené des expériences au moyen d'UMB dans une galette de sable, de 48 cm de diamètre et 28 cm de hauteur, conçue par le Alberta Research Council, dans le but d'étudier la dispersion radiale. Nos expériences ont démontré: une pénétration uniforme et une réanimation des UMB sous le stimulus des éléments nutritifs; les UMB ranimées colmataient de préférence la zone de grande perméabilité en vertu de leur aptitude à croître et à produire un polysaccharide extracellulaire (polymère).

INTRODUCTION

Secondary recovery methods such as water flooding are used to increase oil recovery rates. The waterflood follows the path of least resistance, that is, the high permeability zones, and pushes oil out along that path. These high permeability barren zones now swept of oil are called thief zones as water continues to follow the same course leaving lower permeability zones unswept. A commonly used process to block the thief zones is known as selective plugging. If the particle size of plugging agents is too large, a skin plug develops at the injection well that either prevents injection of water into the unswept formation or the water channels around the shallow plug and is again lost to the higher permeability rock strata. Bacteria were first considered as a selective plugging agent by Crawford (1961). Since that time, many research groups (Jack *et al.* 1983; Jenneman *et al.* 1984; Raider *et al.* 1986) have experimented in their laboratories with vegetative microorganisms. These full size cells stick to surfaces due to their polymer production or are trapped in the pore spaces as described by Shaw *et al.* (1985). To improve the depth of bacterial penetration, we applied a microbial ecology phenomenon first reported by Novitsky *et al.* (1976, 1977). Vegetative cells deprived of nutrients form small (.2 - .3 μm) dormant non-adherent exopolysaccharide free ultramicrobacteria (UMB). When stimulated with nutrients these starved cells grow, reproduce and produce exopolysaccharide (Lappin-Scott *et al.* 1988). Our research group has been experimenting with UMB developed from oil well water isolates as a potential biotechnical process for selective plugging. Initial experiments using scintered glass bead cores, sandpacks and sandstone cores of a variety of lengths and permeabilities confirmed uniform penetration, resuscitation and plugging with UMB (MacLeod *et al.* 1988; Lappin-Scott *et al.* 1988; Cusack *et al.* 1989). Ideally, a field study would prove our process successful, however, field studies are hindered by the complexity of the system, lack of experimental control and time required for measurable results. We decided to use a reservoir simulating system designed by the Alberta Research Council (ARC) in Edmonton, Alberta, Canada. three advantages of this simulator are: 1) it can be packed and simulated to specific field conditions; 2) it is a three-dimensional model allowing for radial flow; and 3) it contains probes strategically located throughout the pack which allows the experimenter to follow the pattern of flow. This paper reports on a selective plugging experiment in a heterogeneous rock strata consisting of a low permeability sand-stone core embedded in a high permeability sand pack.

MATERIALS

UMB and Nutrient:

A *Pseudomonas* species (FC3) was isolated from oil well water and identified by standard microbiological methods. The organism was grown to stationary phase in sodium citrate medium (SCM) containing (gl^{-1} distilled water); $NaC_6H_80_7$, 6.45; $(NH_4)_2SO_4$, 0.198; KH_2PO_4, 2.72; K_2HPO_4, 5.23; $MgSO2_4$, 0.12; $FeCl_3$, 0.008; pH 7.0. The cells were harvested by centrifugation (10,000 x g, 15 min. 4°C) washed three times in a phosphate buffered salts solution (PBS) containing (gl^{-1} distilled water) NaCl, 8.5; KH_2PO_4, 0.42; K_2PO_4, 1.23; pH 7.0, resuspended and starved for 46 days. The final ultramicrobacteria (UMB) solution contained 6.8 x 10^6 cells/ml. The nutrient injected to resuscitate the UMB was SCM.

Large volumes of UMB and SCM solutions were held in sterile carboys until injection. Equipment and/or solutions not sterilized in an autoclave (120°C at 15 PSI for 20-35 min.) were sterilized in a gas sterilizer (Steri-Vac 202, 3M) with ethylene oxide gas for 4 h. On site sterilization consisted of flushing with 5% (v/v) bleach followed by a distilled water rinse.

Equipment as illustrated in Figure 1:

The test simulator is a 45 cm x 38 cm steel cylinder capped at each end with a blind flange. A piston located on top used to apply a 400 PSI (2758 KPA) overburden pressure is separated from the test bed by a wax layer and metal sheets. A saturation distributor located at the bottom is used to saturate the test pack. The injection fluid is injected through a single injection point located at the top centre. Eight sampling probes are located at various points in the pack (Figure 2). Probe locations are such that probes 1 and 2 are closest to the effluent production point, probes 3, 4 and 5 are in the middle, and probes 6, 7 and 8 are closest to the injection point. Differential pressure between the injector and producer is measured using differential pressure transducers calibrated with nitrogen. The pressure and flow rate data are recorded on a Hewlett Packard HP86B computer. The injection fluid is pumped from the reservoir to the test bed with a positive displacement pump (Figure 1) at a flow rate of 120 cc/min. Flow rates are periodically determined from effluent production collection rates. Effluent travels through the producer and is collected in a reservoir where it is sterilized prior to disposal. Effluent samples taken from the production end are labelled as Probe 9 in the results section.

Test bed:

The test bed is made up of a Berea sandstone core (cleveland Quarries, Ohio) measuring 7 cm in diameter x 22 cm in length embedded in +125 mesh Ottawa sand (Figure 3). Porosity of the sand is 40% and permeability is 3.8 darcies. Porosity of the sandstone core is 22% and permeability is 400 millidarcies. The test bed is saturated with filtered sterilized water (.45 μmillipore filter) under vacuum to stabilize mobile fines and to remove air pockets and maintained under an overburden pressure of 400 PSI (2758 KPA).

METHODS

UMB (1.5 pore volumes) are injected from the reservoir to the test bed followed by 1.0 PV of nutrient through the central injector on top of the simulator (Figure 1). Additional nutrient is injected as per schedule shown in Table 1A. Planktonic samples are taken from all probes and the producer (effluent) during UMB and nutrient injection. Each sample is analyzed for viable cell numbers by counting colony forming units (CFU) determined by spread plate dilutions from PBS onto Brain Heart Infusion agar (Difco) incubated at 22°C for 48 h. At 42 days, the test bed is dismantled in four vertical zones. Random sessile samples from each level are removed with a sterile scoop following the grid pattern in Figure 2. The central sandstone core is cut into smaller cores with a drill and bit (Ruska) approximately 5 cm in length. Each small core is cut into smaller sample pieces (A-R in Figure 3), as described by Lappin-Scott *et al.* (1988). All samples are weighed and prepared for viable cell counts, extracellular polymer production (carbohydrate) and scanning electron microscopy (SEM). Sessile viable cell counts are prepared as for planktonic samples. Samples are fixed in 5% (v/v) glutaraldehyde, dehydrated in ethanol (30%-100%) and freon (30%-100%), then coated with gold palladium for SEM. Extracellular polymer production is measured against a glucose standard following methods described by Dubois *et al.* (1956). Samples not processed on site are placed in sterile whirlpack bags (Nasco) and refrigerated until analysis.

RESULTS

Pressure: DP readings increased dramatically from 7.68 KPA to 438.8 KPA during UMB injection. The high pressure reading is likely a direct result of UMB being pushed through the low permeable sandstone core. Injection of the first slug of nutrient reduced the DP to 338 KPA as some of the UMB were dislodged from the test pack. DP readings remained high over the first four weeks, then dropped to 165 KPA over the final two weeks (Table 1A).

Planktonic Samples: Viable cells were found in high numbers in all samples taken from each probe (Table 18). Cell counts of 105 CFU/ml after injection of 1.0 PV of UMB increased to 109 CFU/ml after injection of 6 PV of nutrient over a six week period. Data suggest viable cells are located in the high and low permeable zones in the test bed.

Sessile Samples: Sand and sandstone samples analyzed for viable cell counts and carbohydrate production (Figure 4A, 4B and 5) indicate preferential plugging in the high permeable sand. Sessile cell counts of the high permeability sand samples range from 1.0 x 10^6 CFU/g to 2.1 x 10^8 CFU/g with the lowest counts in level 4 (Figure 4A). Analysis for carbohydrate production showed biopolymer throughout the sand with lowest production in level 4 (Figure 4B). Cells deposited in the low permeable sandstone survived but did not produce large amounts of polymer. Cell numbers ranged from 2.0×10^6 CFU/g to 6.0×10^6 CFU/g, carbohydrate production was less than 60 μg/g throughout (Figure 5). Micrographs (Figure 6) show cells and extracellular polymer in all four levels in the high permeable sand. Few cells and no polymer are visible in the low permeable sandstone.

DISCUSSION

To successfully enhance oil recovery by using microorganisms as a plugging agent, a deep stable plug is required. Moses (1987) thought it necessary to eliminate polymer formation until the bacteria were in the target zone. We now know that penetration of bacteria deep into the reservoir rock matrix can be achieved with UMB. A nutrient injection will "wake up" the UMB and *in situ* plugging will result. Bubela (1983) expressed the importance of reservoir modeling because field studies are limited by a complexity of systems and laboratory studies are sometimes limited by over simplification.

The experiment presented in this paper was developed to use *in situ* nutrient resuscitation of UMB to produce selective bacterial plugging under simulated reservoir conditions in a radial heterogeneous model of high permeability unconsolidated sand and low permeability sandstone. UMB penetrated through the entire test pack resulting in a sharp increase in pressure. Most of the injected nutrients followed the path of least resistance as polymer production was much higher in the high permeability sand pack (10-100 fold). An interesting observation is that a skinplug did not form on the surface of the low permeability sandstone. We conclude that selective plugging occurs *in situ* when UMB are nutrient stimulated. A wide variety of UMB offer an alternative means for plugging high permeability zones to prevent water breakthrough, to prevent coning and to prevent mud loss during drilling operations.

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TABLE 1A

Schedule of Nutrient Injection and Pressure Readings

Day	Nutrient (litres)	Injected (PV)[a]	DP[b] (KPA)
2	15	1	338
7	15	1	303
14	15	1	–
21	15	1	605*
35	15	1	152
42	15	1	165

[a] pv = pore volume

[b] DP = differential pressure

* High pressure is due to plugging of the injection lines.
This was cleared prior to incubation.

TABLE 1B

Planktonic Samples CFU/ml

Sample Probe	UMB 1 PV	SCM 1 PV	SCM 2 PV	SCM 6 PV
1	9.0×10^4	4.4×10^6	3.2×10^8	1.1×10^9
2	3.0×10^5	1.5×10^6	3.2×10^8	1.1×10^9
3	3.2×10^5	2.5×10^6	2.7×10^8	6.6×10^8
4	2.6×10^5	2.5×10^6	2.9×10^8	1.6×10^9
5	2.1×10^5	1.5×10^6	3.7×10^8	1.1×10^9
6	3.2×10^5	1.6×10^6	3.5×10^8	2.3×10^9
7	5.4×10^5	2.7×10^6	3.8×10^8	1.3×10^9
8	2.4×10^5	1.8×10^6	3.2×10^8	2.3×10^9
9	2.0×10^5	1.9×10^6	3.5×10^8	2.6×10^8

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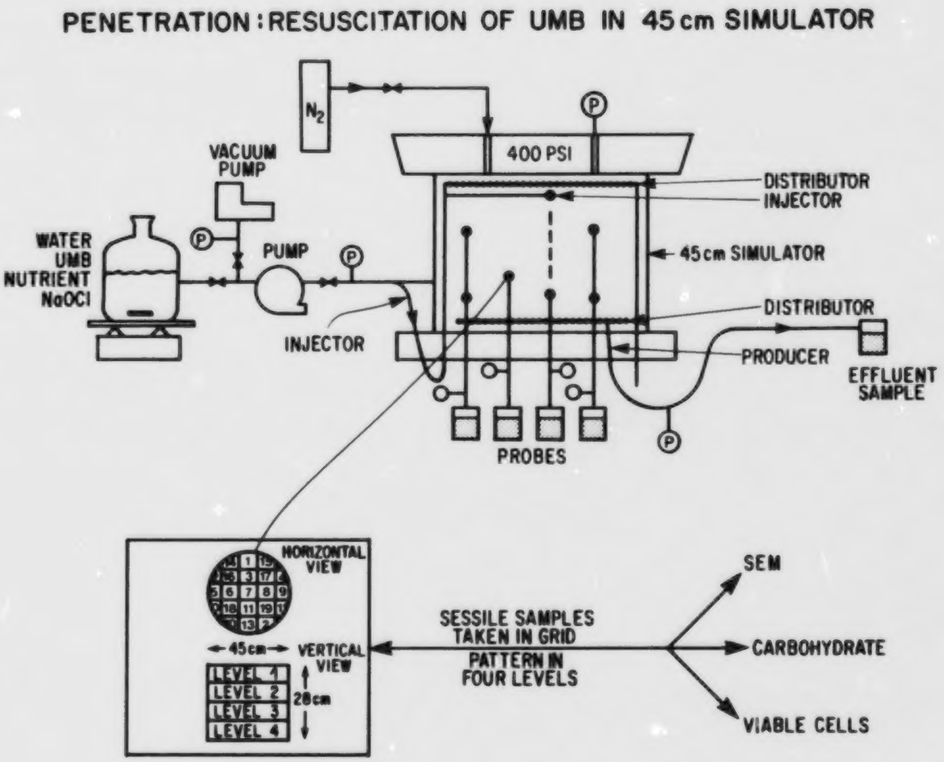
PENETRATION: RESUSCITATION OF UMB IN 45 cm SIMULATOR
N_2
P
400 PSI
DISTRIBUTOR
INJECTOR
VACUUM PUMP
P
WATER
UMB
NUTRIENT
NaOCl
PUMP
P
45 cm SIMULATOR
INJECTOR
DISTRIBUTOR
PRODUCER
EFFLUENT SAMPLE
P
PROBES
HORIZONTAL VIEW
1
3
6
7
8
9
11
19
13
45cm
VERTICAL VIEW
LEVEL 1
LEVEL 2
LEVEL 3
LEVEL 4
28cm
SESSILE SAMPLES TAKEN IN GRID PATTERN IN FOUR LEVELS
SEM
CARBOHYDRATE
VIABLE CELLS

Fig. 1 A schematic diagram of the experimental equipment.

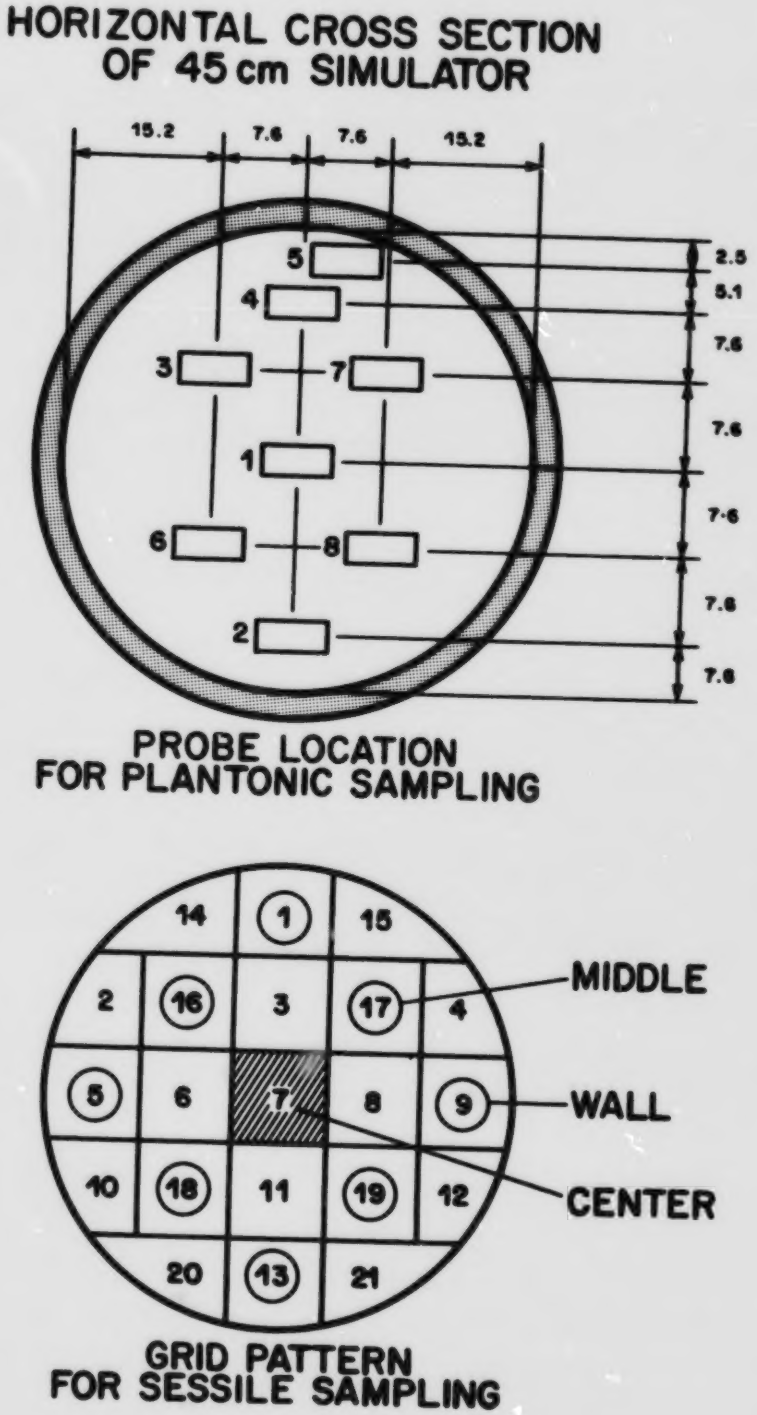
HORIZONTAL CROSS SECTION
OF 45 cm SIMULATOR
15.2
7.6
7.6
15.2
2.5
5.1
7.6
7.6
7·6
7.6
7.6
5
4
3
7
1
6
8
2
PROBE LOCATION
FOR PLANTONIC SAMPLING
14
①
15
2
(16)
3
(17)
4
⑤
6
7
8
⑨
10
(18)
11
(19)
12
20
(13)
21
MIDDLE
WALL
CENTER
GRID PATTERN
FOR SESSILE SAMPLING

Fig. 2 A cross-sectional view of the 3-dimensional sand pack showing the planktonic sample probe locations and the grid pattern for sessile sampling.

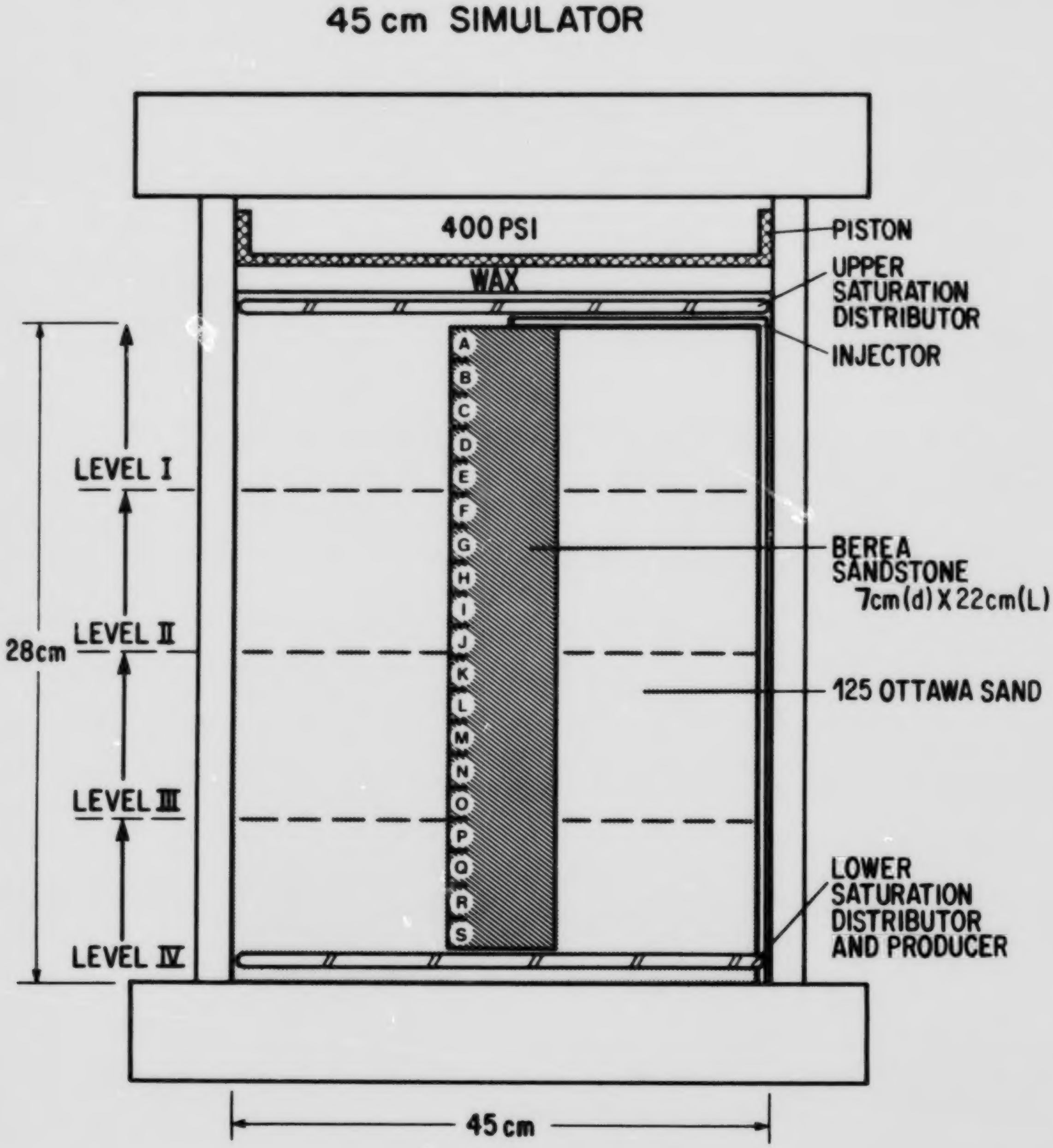
45 cm SIMULATOR
400 PSI
PISTON
WAX
UPPER SATURATION DISTRIBUTOR
INJECTOR
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
LEVEL I
LEVEL II
LEVEL III
LEVEL IV
28cm
BEREA SANDSTONE 7cm(d) X 22cm(L)
125 OTTAWA SAND
LOWER SATURATION DISTRIBUTOR AND PRODUCER
45 cm

Fig. 3 Sandstone core and sand pack in the 45 cm simulator. A-Q are locations of sandstone samples.

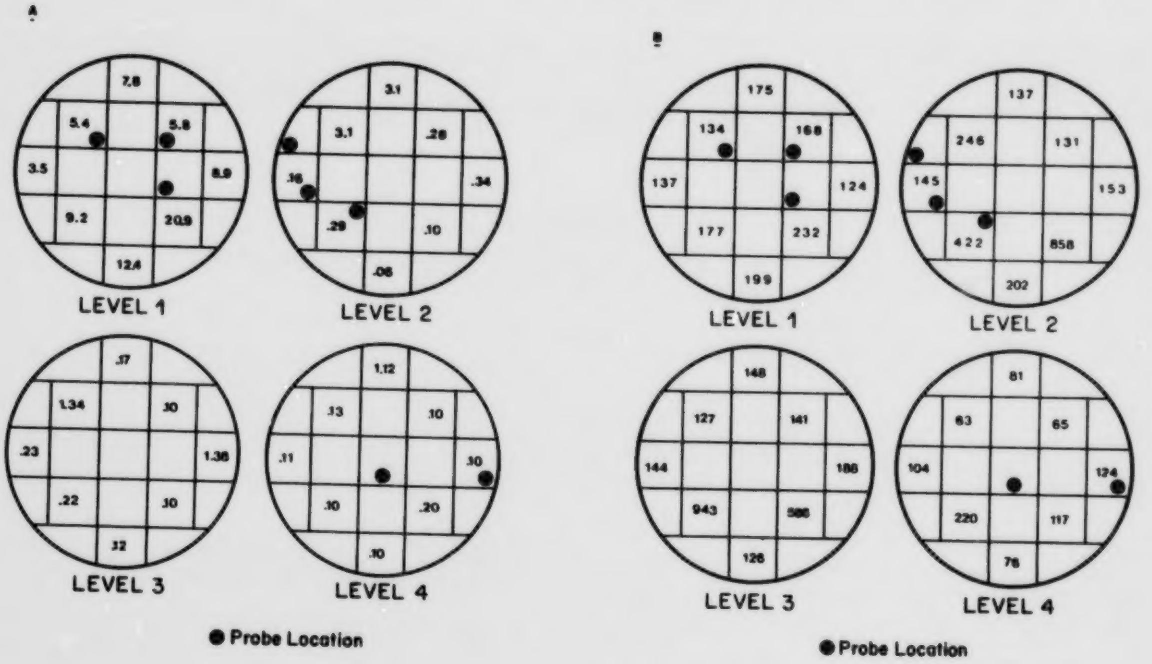
A
7.8
5.4
5.8
3.5
8.9
9.2
20.9
12.4
LEVEL 1
3.1
3.1
.28
.16
.34
.29
.10
.06
LEVEL 2
.17
1.34
.10
.23
1.38
.22
.10
.12
LEVEL 3
1.12
.13
.10
.11
.10
.10
.20
.10
LEVEL 4
● Probe Location
B
175
134
168
137
124
177
232
199
LEVEL 1
137
246
131
145
153
422
858
202
LEVEL 2
148
127
141
144
188
943
586
128
LEVEL 3
81
63
65
104
124
220
117
78
LEVEL 4
● Probe Location

Fig. 4 Diagrammatic representation of (A) viable cell counts (CFU/g x 10^7) and (B) carbohydrate production (μg/g) of the attached sessile population in the heterogeneous test bed.

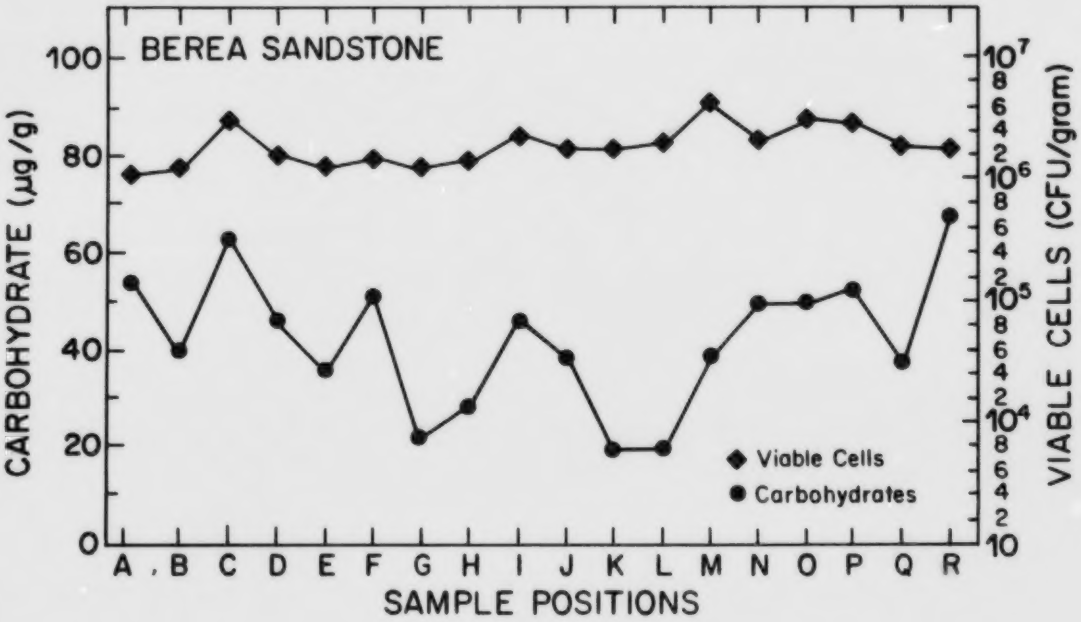

Fig. 5 Graphic representation of viable cell counts (°) and carbohydrate production (Δ) of the sandstone sections from the central low permeability sandstone core in the heterogeneous test bed.

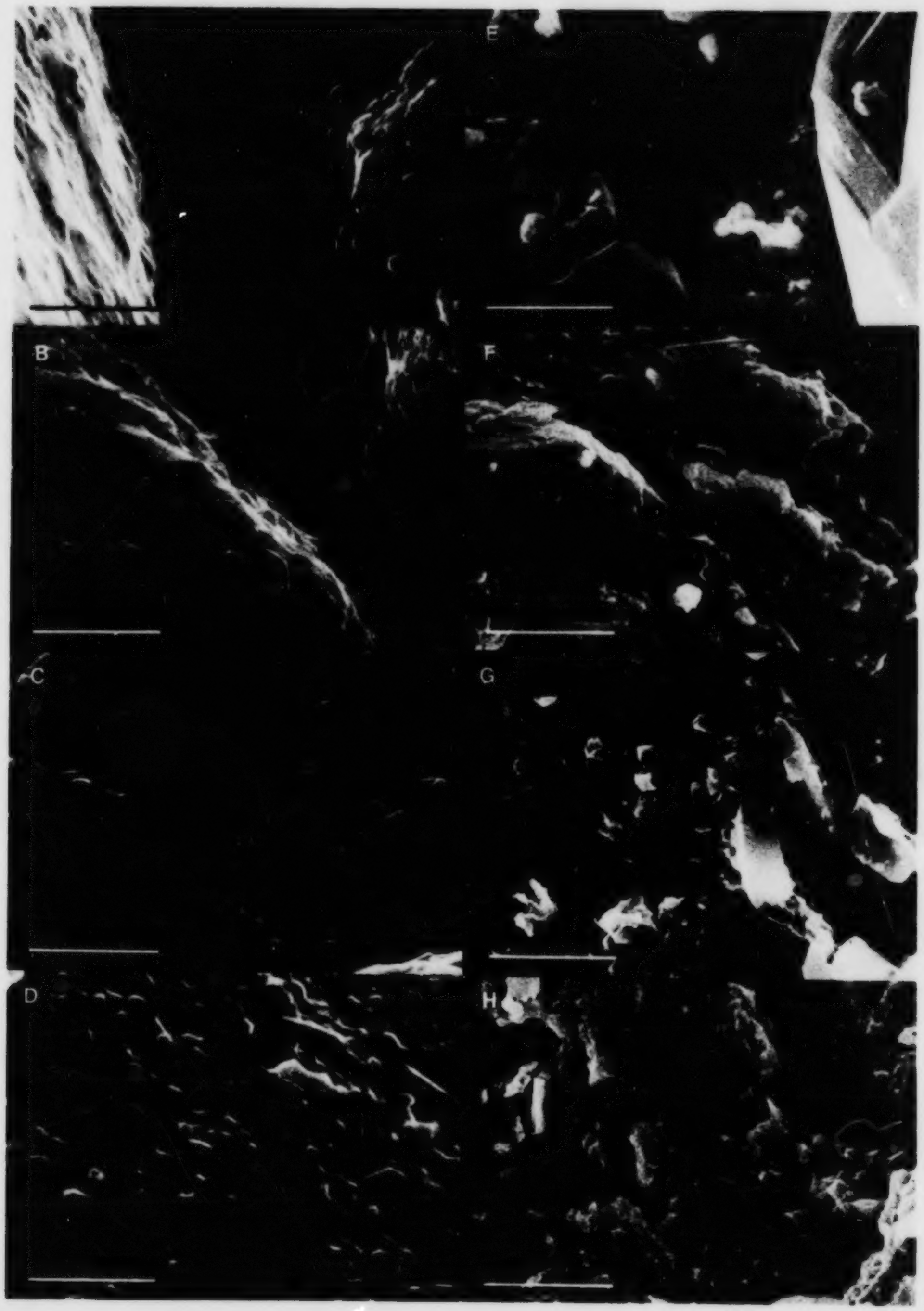

Fig. 6 Scanning electron micrograph of four samples taken from the sand pack, A-D, and four samples of the sandstone core, E-H. Bacterial polymer is in each of the high permeability sand pack sections but not in the low permeability sandstone sections showing selective plugging of the high permeability zones. Scale bar represents 5 μm.

FIELD TEST OF A MICROBIAL PLUGGING SYSTEM AT STANDARD HILL, SASKATCHEWAN TO INHIBIT OILFIELD WATER ENCROACHMENT.

L.G. Stehmeier, T.R. Jack, B.A. Blakely,*

and

J.M. Campbell

Nova Husky Research Corporation,
2928 - 16th Street N.E., Calgary,

and

Husky Oil Operations Ltd., 707 - 8th Avenue S.W.,
Calgary, Alberta, Canada.

ABSTRACT

A field test of the NOVA HUSKY RESEARCH CORPORATION (NHRC) microbial plugging system was carried out in heavy oil fields in Western Canada. This field test was an attempt to seal off an aquifer that progressively moved across a field causing 100% water production. The field test consisted of laboratory studies on the enhancement of bacterial dispersion, a radioactive tracer survey of the reservoir to describe the target field, use of the laboratory studies to optimize field injection of the NHRC microbial plugging system, and monitoring the field to determine the results of the test. The microbial system was swept more than one kilometer from its injection point into the reservoir where growth of the injected bacteria was measured. Overall pressure drawdown tests failed to show significant plugging in the throat of the aquifer where injection had taken place.

ESSAI SUR LE TERRAIN D'UN SYSTÈME DE COLMATAGE MICROBIEN À STANDARD HILL, SASKATCHEWAN, POUR INHIBER L'ENVAHISSEMENT PAR L'EAU D'UN CHAMP PÉTROLIFÈRE

L.G. Stehmeier, T.R. Jack, B.A. Blakely*

et

J.M. Campbell

Corporation de Recherche Nova Husky, Calgary

et

l'Opération Pétrolifère Husky Ltée, Calgary (Alberta), Canada

RÉSUMÉ

Au début des années 1980, Nova Husky Research Corporation (NHRC) a mis au point un système microbien pour colmater de façon sélective les zones aquifères dans les grands champs pétrolifères de l'ouest du Canada. L'infiltration d'eau se traduit par des coupures d'eau coûteuses, ce qui se produit normalement au moment de l'extraction primaire aux puits de pétrole brut. Un essai sur le terrain, constitué d'études en laboratoire, des problèmes d'injection et de dispersion des bactéries dans le réservoir cible, d'une description du réservoir du champ visé et de l'injection du système de colmatage microbien mis au point par la NHRC, a été mené conjointement par la NHRC et la Husky Oil Ltd. D'après les données connues de ce champ, on trouve des puits qui, en séquence, frappent de l'eau dans une direction sud-ouest, ce qui indique la présence d'un envahissement par une nappe aquifère. Des éléments traçants radioactifs ont permis d'identifier le point d'entrée de l'eau provenant de cette nappe aquifère et le système de colmatage a été injecté à cet endroit.

INTRODUCTION

NOVA HUSKY RESEARCH CORPORATION has developed and patented a novel microbial selective plugging system for profile correction in heavy oil fields [Thompson and Jack (1985)]. The system uses bacteria able to grow *in situ* in the target reservoir on injected fermentable nutrients. The bacteria are introduced as an aqueous slurry which preferentially enters zones or channels of high water permeability. Once in place they are overrun by a nutrient package containing a trigger compound which initiates insoluble polymer production in situ plugging off the affected zone. The prime aim is to shut off undesirable water flow. In the case of the present field test, a strain of *Leuconostoc mesenteroides* was employed with sucrose as the trigger compound.

This paper summarizes the results of a jointly funded field test of this system with Husky Oil and Canmet, EMR under the Extraction and Recovery of Bitumen and Heavy Oil Subprogram, Dr. Albert George, assigned scientific officer.

Results will be discussed under three principal headings; laboratory studies concerned with the problems of injection and dispersion of bacteria in the target reservoir, a description of the field target and finally the field test results.

LABORATORY STUDIES ON BACTERIAL INJECTION AND TRANSPORT

The selective plugging scheme depends on the successful injection of appropriate bacteria into the reservoir [Thompson and Jack (1985)]. This entails two challenges: maintaining injectivity as the bacterial suspension enters the formation face and obtaining adequate dispersion or travel through the target information.

Injectivity can be lost if the formation face acts like a filter becoming clogged with bacterial cells, polymers or other debris. Past work indicates the bacterial cells chosen must be free of exopolymers, not producing gas bubbles and as small as possible with respect to the pore size distribution in the host formation [Jack *et al.* (in press)]. Small cocci [Thompson and Jack (1985)], spores [Jack and Thompson (1983)] and ultra-microbacteria [Costerton *et al.* (1988)] have all been considered suitable. Severe facial plugging can reduce the permeability of the formation face to the point where further injection becomes impractical because of high pressure build up. This is not irreversible damage. Strong oxidants [Crowe (1969) and Cusak *et al.* (1987)], chelants [Turakhia *et al.* (1983)] and reperforation of the well [Jack and Stehmeier (1988)] can all restore injectivity.

The main concern of this project was not injectivity *per se* but dispersion of the bacteria deep into the reservoir. Undue retention or adsorption of bacterial cells during travel through the formation will limit the distance which cells can be injected into a water zone or channel system.

Work to date has either failed to differentiate injectivity and dispersion [Postgate (1984), Shaw (1982), Jang *et al.* (1983), Sharma *et al.* (1985)] or has been based on the diffusion or growth of bacterial cells through rock matrices under very low pressure differentials [Jenneman *et al.* (1985), Myers and McCready (1966)]. While the latter studies indicate that bacteria can penetrate remarkably tight (low permeability) materials, the rates involved are too slow to be the sole means of placing a MEOR system in a field application. Rapid penetration of bacteria through reservoir rock, necessary for most applications, will require the bacteria to be suspended in a carrying agent such as water.

Very little is presently understood about the factors which control bacterial travel through a geological matrix under injection. This information would obviously be of great value both in placing MEOR systems of all sorts in target reservoirs and in considering various bacterial souring and plugging problems which plague certain field operations such as waterflooding.

In 1985 a cursory study at NHRC discovered that trace clays could adsorb and significantly retain cells of *Leuconostoc mesenteroides* during injection through model sandpacks in the laboratory. In a preliminary study (Figure 1), the rate and extent of cell breakthrough was greatly enhanced by pretreating sand cores with a certain proprietary sacrificial agent developed at B.C. Research with EMR funding [Howard and Stirling (1987)]. This sacrificial agent will coat the clays and fines found in the reservoir which normally present a large area for bacterial adsorption. Coated with sacrificial agent, this surface area is masked and bacterial adherence is decreased.

To elucidate the factors controlling transport of injected *L. mesenteroides* cells through sand reservoirs, a series of twenty-two core runs were made injecting bacterial suspensions under various conditions through sand packs made from sands taken from oil reservoirs in the Lloydminster region.

A new core was prepared for every test. Sand and core dimensions were varied during the tests. Permeabilities ranged from < 1 to 6.5 darcies. Each core was washed with brine overnight at a constant flow rate of 1 ml/minute. In selected cases, various amounts of sacrificial agent were injected followed by a saline rinse until the effluent was a pale yellow, indicating there was no unadsorbed sacrificial agent remaining in the core. Cells suspended in sterile 0.8% NaCl, pH 7, at concentrations of 10^7 to 10^8/ml were injected for 21 to 92 pore volumes at flow rates from 10 to 110 cm/hour. The effluent from the cores was collected automatically in 10 ml fractions and assayed for bacteria. The injection pressure was monitored as an indication of permeability loss.

The results of these studies indicate that the factors controlling bacterial travel are complex. The experiments were complicated by plugging effects arising from fines migration in cores using whole unsorted sand and by growth of *competing bacterial species in* some cases. Cores showing plugging due to extensive contamination or fines migration failed to allow transmission of cell suspensions of *L. mesenteroides* over distances of 92 cm even after 31 or more pore volumes throughput.

Despite these difficulties, successful core runs were achieved and a number of interesting observations were made.

The quantity of cells injected was linearly related to the number of cells emerging from a sandpack. Figure 2 shows the results for a number of sandpack experiments with and without sacrificial agent pretreatment. The beneficial effect of the sacrificial agent in improving transmission can be seen to increase the slope of the plot threefold but about one half the cells are still retained. The absence of plugging in these cores suggests that this retention is not simply due to formation of a filter cake on the face of the core.

Flow velocity was another important parameter. Those cores having the highest flow rates (1.1 m/hour) consistently gave high, fast cell throughputs. When the *linear* velocity was dropped to 39 to 55 m/hour, results became variable with some cores showing slow cell breakthrough and high retentions while others showed no cell breakthrough at all even after more than 29 pore volumes, again without an appreciable loss of permeability. Possibly these lower flow rates are in a critical range in terms of maintaining cells in suspension during travel through the porous medium.

A significant fraction of the cells retained in a sandpack can be displaced by pulsing the injection of fluids through the core. (In Figure 3 ninety pore volumes of bacteria were injected into the column for a total of 1.3×10^{12} bacteria. After the last bacterial injection, the core was flushed with 0.8% saline until the bacterial effluent concentration had dropped to $< 10\%$ of the injected level. The pump was then shut off allowing the injection pressure to drop to one-hundreth its original value. The pump was then turned on again for one pore volume and the process repeated ten times. The cells washed out of the core by these ten pulses amounted to 1.35×10^{11} cells or 10.5% of the total cells injected. This compares to 33.7% cell passage during the previous 139 pore volumes. The number of cells per spike seemed to drop off over the sequential pulses (Figure 3) indicating that the number of cells which can be mobilized in this way may be limited. The cells displaced by this technique peaked early in each pulse. The displaced cells could not, therefore, be coming from the front face of the core (from a facial plug, for example) but must be dislodged from retention sites within the core itself. Careful analysis suggested cells are mobilized and travel through less than one-third of the core before being retained again. These observations are consistent with the formation of "log jams" of cells in pore throats (held in place by sustained flow.)

These lab tests indicated that the main obstacle to placement of a satisfactory microbial plug in the field would not be a loss of injectivity due to facial plugging of the formation during injection of the bacterial cells but deep penetration of the cells into the reservoir. It was decided to use a sacrificial agent and pulsed injection in the field to move the bacterial system away from the injection well and deep into the formation to be plugged before introducing the nutrient package which would trip insoluble polymer formation *in situ*.

THE FIELD TARGET

A major problem in heavy oil fields is the competitive production of water. Due to the large difference in mobility between water and oil, relatively small "channels" of high water permeability result in the overwhelming production of water ultimately "watering out" the heavy oil well. This field test was an attempt to plug such a channel in a heavy oil field on primary production.

The Standard Hill Pool produces from the "A" zone of the Lower Waseca formation of the Lower Cretaceous Mannville Group. The Lower Waseca formation is a stacked sequence of coarser mudstones to very fine grained sandstones. The layer is typically 24 m thick with a 0.5 m cap of coal. The "A" zone is the uppermost cycle in the sequence and averages 3.5 m of porous sand distributed as northeast trending bar-forms ranging from 2.0 m to 7.5 m in thickness. The "A" zone is interpreted to represent a shoreface depositional setting. Density log porosities are from 33% to 35% and core air permeabilities are in the range of 2 to 5 Darcies. Drilling depths are in the order of 460 m. Oil is structurally trapped by regional tilt to the southwest and a salt collapse low to the north east. Structure on the top of the "A" zone ranges from a high of 132.0 m above sea level in the northeast to 96.0 m *above sea level in the southwest.* The oil/water contact is at 96.6 m above sea level.

In 1981 after producing only 200 m^3 of oil, the well A9-2 (Figure 4) at the edge of the field broke to water. In February 1983, the well B8-2 broke to water after producing 7300 m^3 of oil while three offset wells, A2-2, A7-2 and A10-2 watered out in the first quarter of 1983. All had produced significant quantities of oil before watering out. From 1983 to 1988 nineteen other contiguous wells broke to water. The sequence of wells was not based on the movement of water to the lowest structural point in the reservoir, *but did follow a southwest trend across* the field. This pattern led to the hypothesis that a "water channel" had developed from the aquifer into the oil pool. The good oil production characteristic of this field warranted its selection as a target for the microbial plugging system with the aim of slowing the south westerly invasion of water.

Well A9-2 was thought to be the entree well for the channel into the field because it had been the first to water out. Based on this theory, well A9-2 was recommended as the application point for the plugging system. Before proceeding however, an interwell tracer study was performed using tritiated water to delineate the channel system.

The first tracer survey showed well A9-2 was in fact not the source of water entry into the Standard Hill Field. No indication of tritium was picked up in the offset wells and a flowback test produced significant quantities of tracer remaining in the well after several weeks.

The second tracer survey at well B8-2 showed a network of highly permeable water channels between well B8-2 and the offset producing wells. The cumulative tracer recovered from the offset producers was estimated to be between 6.9% and 17% of the original amount injected. Total channel volume, estimated from the recovery and flow velocity of the tracer, was 500 m^3. The channel from B8-2 to

A7-2, which gave apparent flow velocities of 97 m/hr, was estimated to have a volume of only 50 m^3 [Blakely (1988)].

Based on these results, well B8-2 was identified as the injection point for the microbial plugging system. The intention was to cause plugging of the channel system close to its inlet point into the field to slow the rate of water influx and consequent loss of wells to water deeper in the field.

FIELD TEST RESULTS

Figure 5 is a schematic diagram of the field injection equipment.

Despite the remarkable flow rates seen in the water channel, sacrificial agent was injected prior to the bacterial slurry to minimize the number of bacteria retained near the injection well and to ensure deep penetration of the bacterial system into the channel system. Bleach was used to prevent growth in the well bore itself to keep the formation face clear for subsequent injection of nutrients.

The inoculum, grown on a medium free of sucrose and thus unable to support polymer formation was prepared at Alberta Distillers Limited, Calgary. The inoculum was scaled up in three steps from 10 mL of 2 x 10^8 cells/mL to 20000 L of 1 x 10^8 cells/mL, an optimal concentration based on lab studies [Stehmeier and Jack (1987)]. The inoculum was trucked to the site in disinfected tank trucks.

The sacrificial agent which is an amended pulp mill effluent was trucked to the site from the Weldwood Champion Forest Products mill in Hinton, Alberta. In this sacrificial agent there is a quantity of fibrous particles capable of plugging the face of 1 Darcy sand packs [Stehmeier (1987)]. A filtration scheme (Figure 5) developed to remove particulates $>0.8\mu$ in the field test but failed rapidly. Filtration was continued at a larger retention size (3μ) without increase in injection pressure. This indicates the reservoir face is not as tightly packed as the lab sand pack and, for Lloydminster unconsolidated sand reservoirs, expensive submicron filtering may not be necessary for such channel systems.

The injection of the bacterial slurry went smoothly and was followed by four 5 m^3 pulses of fresh water to enhance bacterial travel into the reservoir. Only two wells were left pumping throughout the injection process, A15-2 and A3-2. This was to ensure that the injected materials moved down the channel into the reservoir rather than out into the aquifer. The actual flow rate in the channel during the injection was not known.

Immediately after injection of the sugar beet molasses medium, samples from these wells contained 1% sucrose and showed at least fifty fold increase in the number of polymer forming bacteria present. The results of an identification procedure, based on the fatty acid methyl ester profile of bacteria, showed that an isolate from well A15-2 was identical to the injected bacteria. *In baseline studies Leuconostoc were not found to be indigenous to the reservoir.*

Production of selected wells was resumed after a two week incubation period to allow full polymer development.

Coproduced water from well A6-35 showed high levels of metabolites characteristic of *Leuconostoc mesenteroides* (Figure 6). The presence of excess fructose provides substantial but indirect evidence of insoluble polymer formation in situ. This polymer is a dextran made via the enzyme dextran sucrase which splits the disaccharide sucrose incorporating the glucose moiety into a growing polymer and releasing free fructose into solution. The presence of excess fructose, a sugar easily degraded by other organisms, also indicates that the injected *Leuconostoc* was the predominant bacterial species in the fermentation. Plate counts on a molasses agar medium showed polymer forming bacteria were present at a concentration of more than 10^6 colony forming units per mL in the effluent from this well. The identification procedure described above identified isolates as being indistinguishable from the injected culture. The distance travelled in this example is approximately 1.8 kilometres or greater than 100 m/day assuming straight line travel between B8-2 and A6-35 (Figure 4).

Injected bacteria were also identified at A1-3, A3-35, A5-35, A11-35, A10-34 and A16-34 but neither the cell counts nor the metabolite analyses indicated the high level of *in situ* growth and metabolism seen at A6-35. The general presence of slime forming bacteria in the produced water after injection and their absence during baseline studies prior to the field test suggest polymer formation took place in the reservoir generally. After approximately two weeks, *bacterial counts and metabolic product concentrations began to decrease indicating that the* bloom of activity caused by the injected molasses was subsiding.

Water analyses indicated changes did take place in the reservoir after injection of the bacterial plugging system. The pH changes seen were not large, except for isolated wells, which suggests pockets of microbial activity. Examples of this would be A6-35 and A12-35. This isolated pattern indicates that bacteria and metabolites were not distributed evenly throughout the channel system. Conductivity measurements were used to track the injected slugs since all injections were made up with essentially fresh water and the connate water in the field is a brine. (*Up to a five fold freshening of the produced water was seen at diverse wells at different times suggesting fresh water was traveling in slugs through a low volume channel system.*) Some wells showed cyclical freshening indicating that the channel system perhaps resembles a spider's web in which the fresh water slug was split up and flowed through different routes to the same well to arrive at different times.

Overall pressure drawdown tests conducted on the whole field failed to show significant plugging. Water influx rates were not substantially abated and the overall conclusion was that while polymer plugging could have occurred at isolated points deep in the channel system, no significant plugging was achieved in the throat of the channel system near the injection well B8-2. Indeed, the occurrence of the injected organisms deep in the field and evidence of the desired metabolism at well A6-35 indicate that the bacterial system was flushed more than a kilometer from the injection point. The test was quite literally a washout. The low volume, high velocity and complexity of the channel system evident in these results was unanticipated and prior concern about the ease of bacterial injection and transport in these reservoirs was clearly unwarranted.

SUMMARY

Evidence of in situ microbial activity, resulting from injection, has demonstrated the possibility of using a microbial plugging system in heavy oil reservoirs. The pattern and composition of well fluids after injection indicate a much more complex flow in the reservoir than was expected. A low volume high velocity spider's web complex of channels is suggested. Failure to plug off the water influx in this test suggests extensive work defining the target for plugging systems needs to be done. The results also suggest concerns about limited bacterial transport may be greatly exaggerated in this kind of application.

ACKNOWLEDGEMENTS

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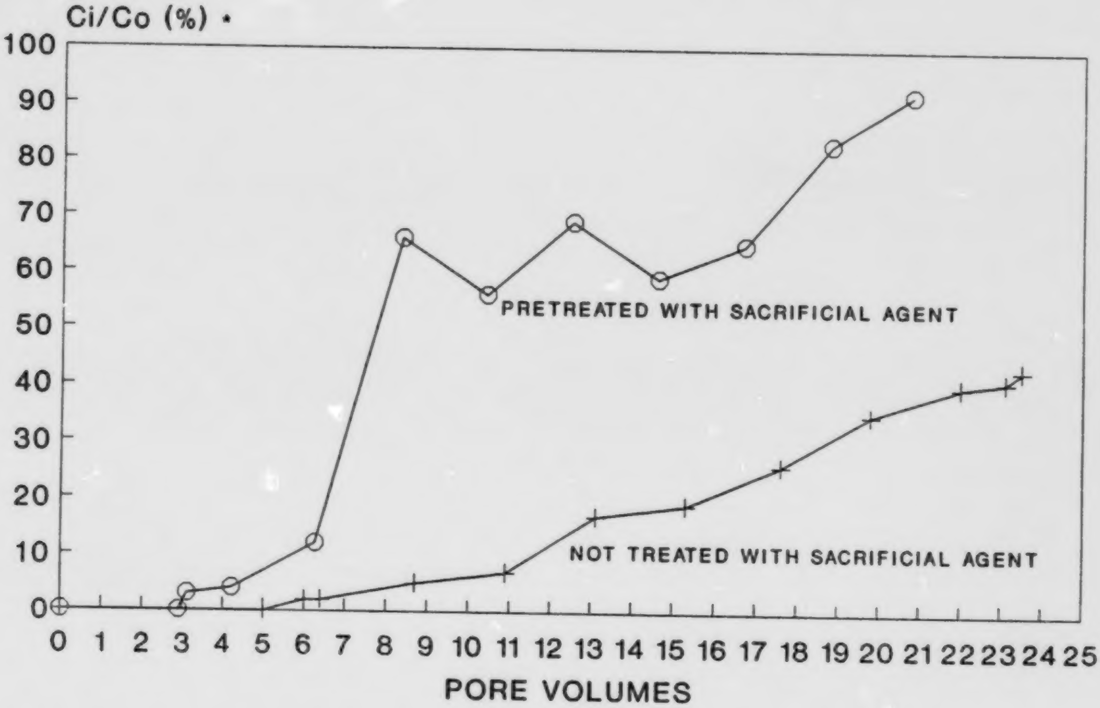
FIGURE 1 BACTERIAL EFFLUENT FROM SAND PACKS WITH AND WITHOUT SACRIFICIAL AGENT TREATMENT



$$* \ \mathrm{Ci/Co}\ (\%) = \frac{\text{EFFLUENT CELL CONCENTRATION}}{\text{INITIAL CELL CONCENTRATION INTO THE SANDPACK}} \times 100$$

FIGURE 2 EFFICIENCY OF CELL TRANSMISSION THROUGH SANDPACKS

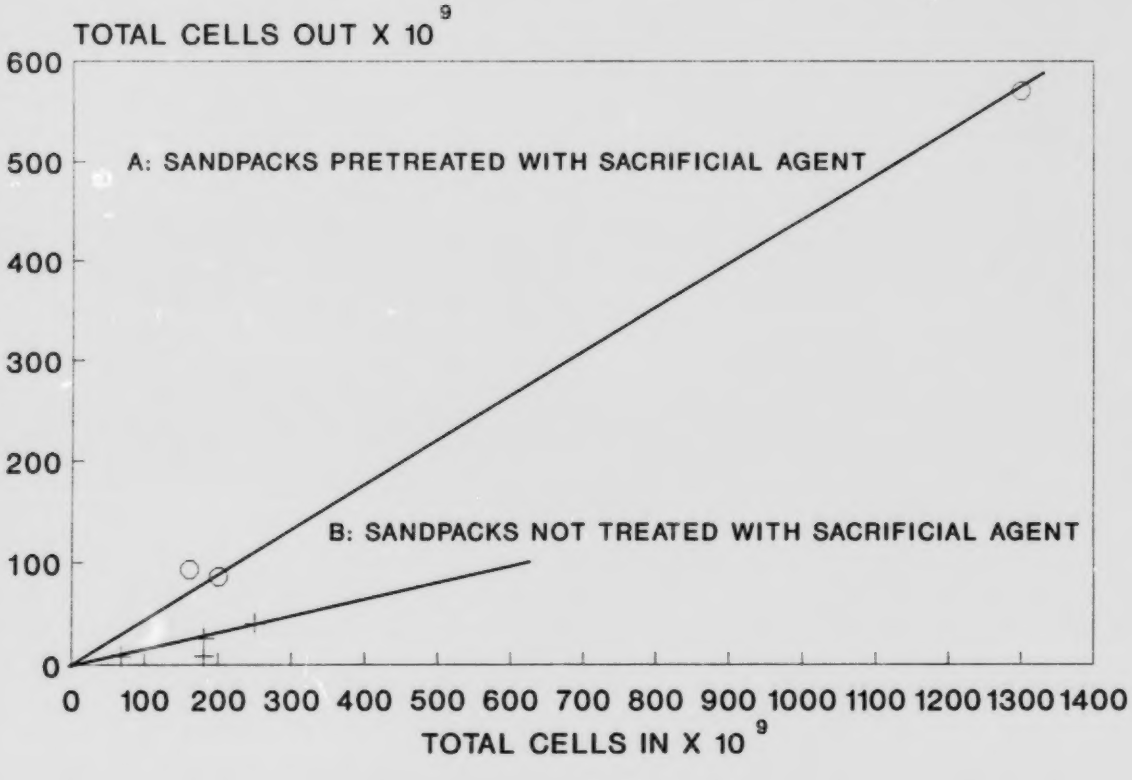
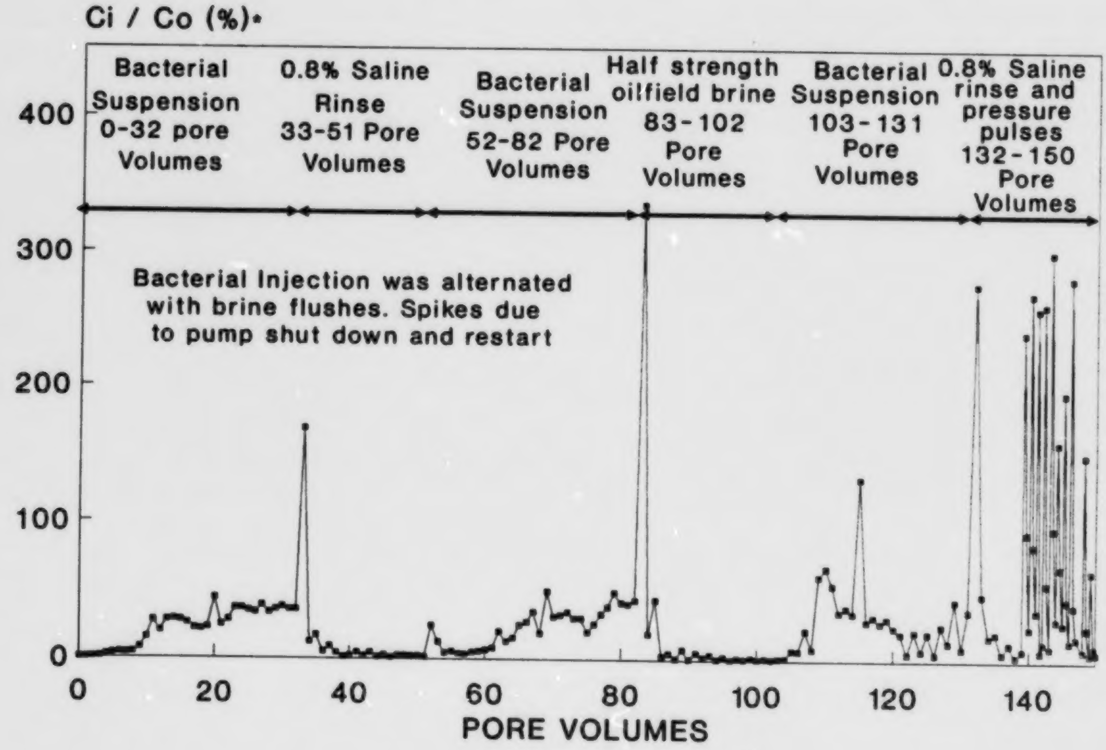


FIGURE 3 DISPLACEMENT OF RETAINED BACTERIA FROM A 37 cm CORE BY PRESSURE PULSATIONS



$$* \; Ci/Co\ (\%) = \frac{\text{EFFLUENT CELL CONCENTRATION}}{\text{INITIAL CELL CONCENTRATION INTO THE SANDPACK}} \times 100$$

Figure 4 WELL LOCATION IN THE STANDARD HILL FIELD, HUSKY OIL LTD.

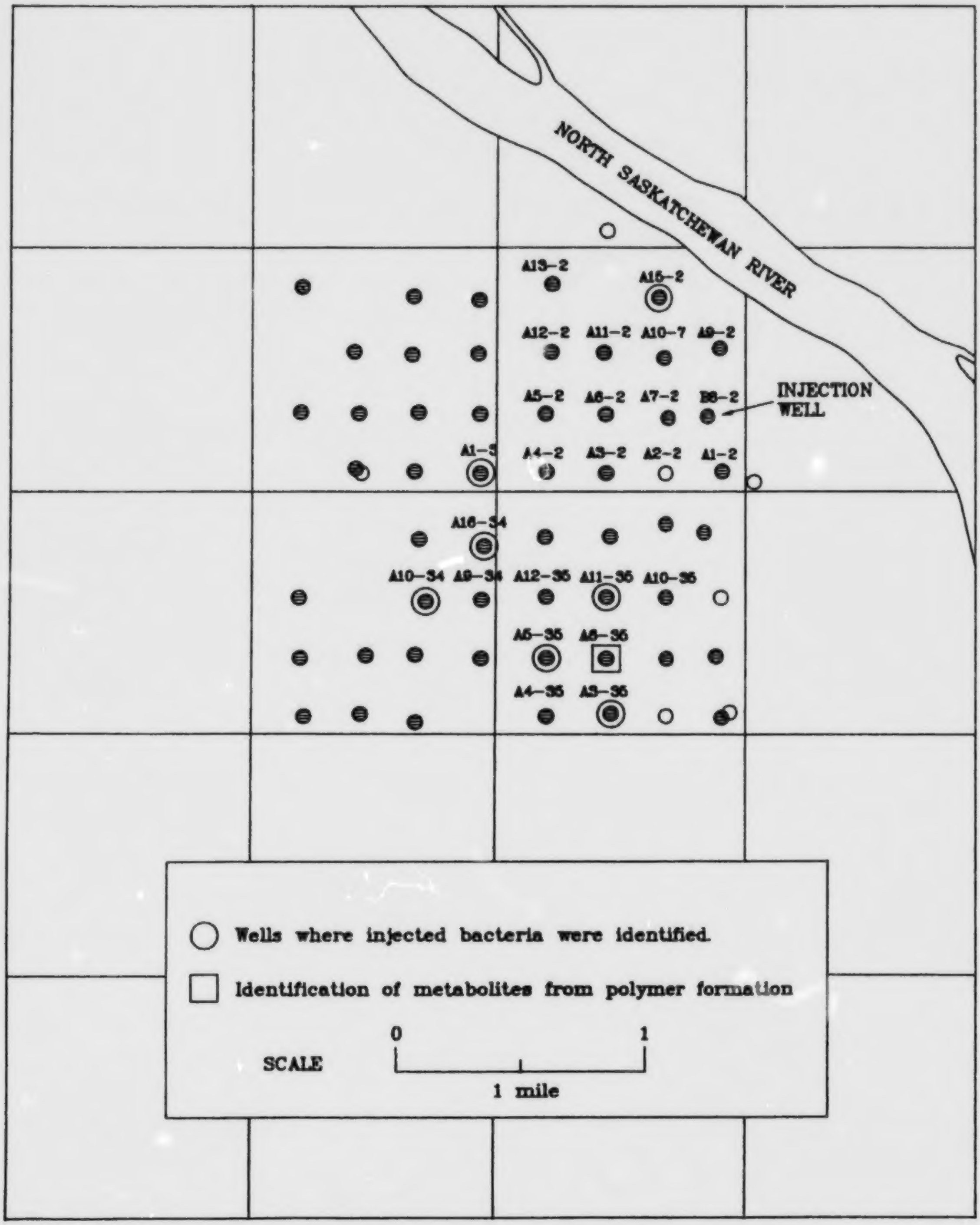

NORTH SASKATCHEWAN RIVER
A13-2
A15-2
A12-2
A11-2
A10-7
A9-2
A5-2
A6-2
A7-2
B8-2
INJECTION WELL
A1-3
A4-2
A3-2
A2-2
A1-2
A16-34
A10-34
A9-34
A12-35
A11-35
A10-35
A5-35
A6-35
A4-35
A3-35
○ Wells where injected bacteria were identified.
☐ Identification of metabolites from polymer formation
SCALE
0
1
1 mile

FIGURE 5: SCHEMATIC DIAGRAM OF FIELD INJECTION SYSTEM

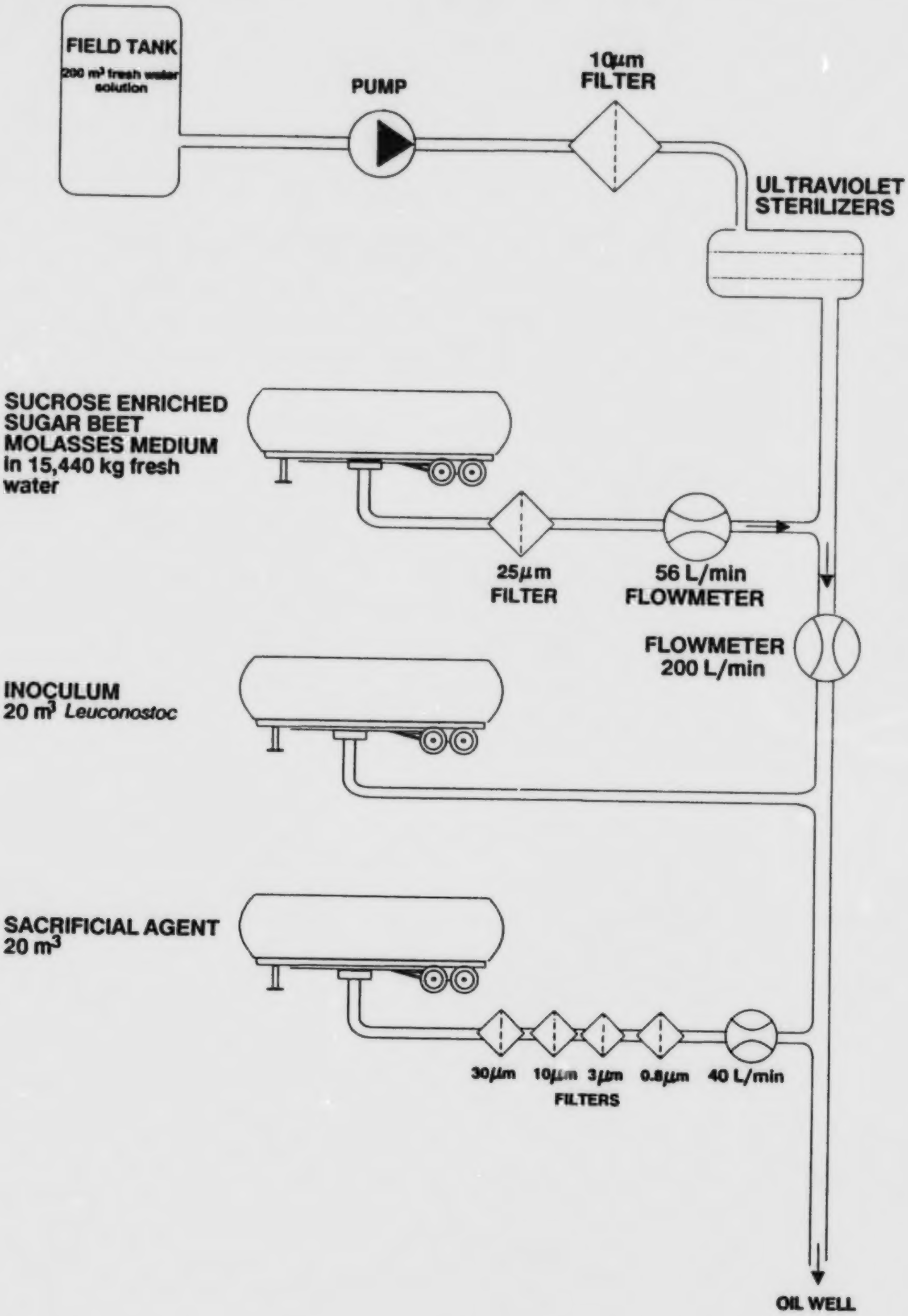
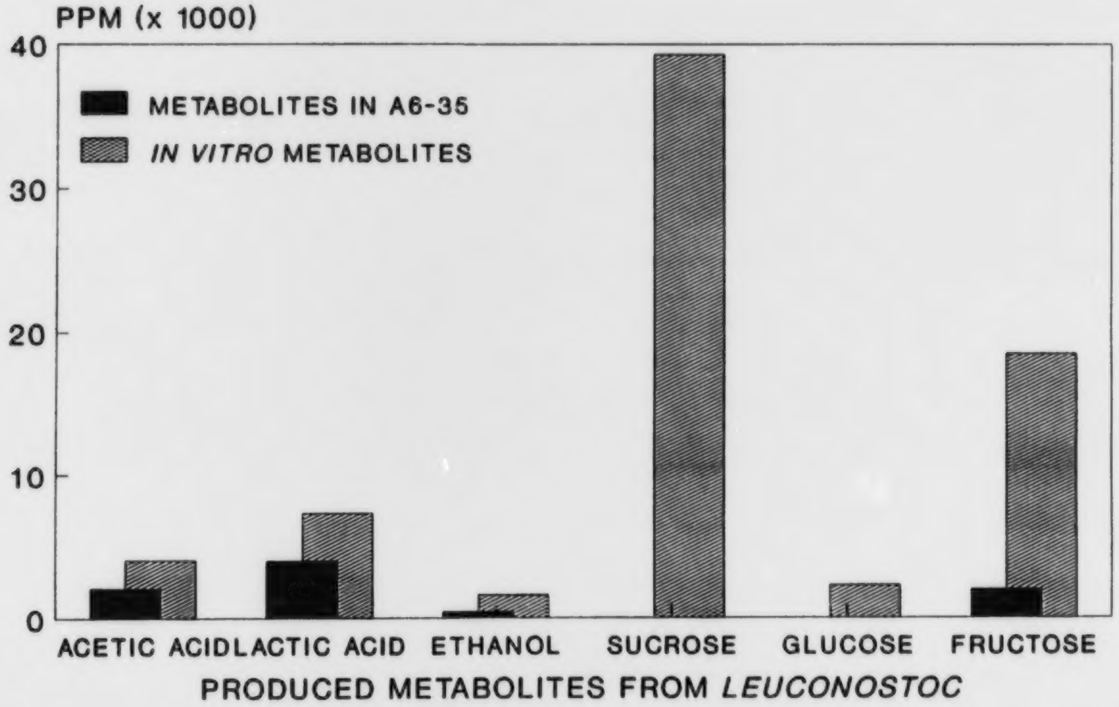

FIELD TANK
200 m³ fresh water solution
PUMP
10µm FILTER
ULTRAVIOLET STERILIZERS
SUCROSE ENRICHED SUGAR BEET MOLASSES MEDIUM in 15,440 kg fresh water
25µm FILTER
56 L/min FLOWMETER
FLOWMETER 200 L/min
INOCULUM 20 m³ Leuconostoc
SACRIFICIAL AGENT 20 m³
30µm
10µm
3µm
0.8µm
FILTERS
40 L/min
OIL WELL

FIGURE 6 COMPARISON OF METABOLITES PRODUCED IN THE LAB AND IN WELL A6-35 ON AUGUST 15, 1988



COAL DESULFURIZATION BY BIOHYDROMETALLURGICAL PROCESSING: A PROGRESS REPORT ON A THREE-YEAR PROJECT

M. Agus(1), *C. Conti*(2), *N. Orsi*(2), *V. Pietropaolo*(2), *G. Rossi*(3),
P. Tataseo(2), *P. Trois*(3) *and P. Valenti*(4)

ABSTRACT

A considerable part of the world's mineable coal reserves is characterized by sulfur contents well in excess of the limits established in most countries by environmental protection regulations. The development of effective sulfur removal processes from coal might therefore drastically increase energy resources. This paper is a progress report on a research program by an Italian multidisciplinary team. Four European coals, ranging in total sulfur content from 1.6% to 6.0%, with varying proportions of organic sulfur, are being investigated. Up to 90% pyritic sulfur removal can be achieved by microbial mediation provided that coal is adequately comminuted and the suspension medium ingredients are consistent with the coal's chemical composition. Highly porous coals are the most readily amenable to biodepyritization.

Microbial strains isolated from enrichment cultures contained by repeated samplings in an Italian underground sub-bituminous coal mine are identified as *Bacillus* sp., *Enterobacter* sp., *Micrococcus* sp. and *Pseudomonas* genus.

The ability of strains of *Pseudomonas* genus to degrade sulfur compounds was tested using dibenzothiophene (DBT) as a model molecule. This compound was added to minimal media as sole sulfur source, in addition to Na benzoate (NaB) as sole carbon source, or as sole sulfur and carbon source. Some strains are able to remove more than 40% organic sulfur and evidence was provided, by auxanographic tests, that the final degradation product is sulfate ion and that this oxidation occurs by direct contact of the microorganism's cell wall with the chemical compound. The adapted strains were also tested for organic sulfur removal activity from an Italian coal and were found to be capable of removing up to 18% of organic sulfur.

On the grounds of the observations made, a flowsheet is proposed for a pilot plant for microbial coal desulfurization.

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(1) Italian National Research Council

(2) Institute of Microbiology, "La Sapienza" University, Rome, Italy

(3) Mining and Mineral Dressing Department, University of Cagliari, Italy

(4) Institute of Microbiology, 1st Faculty of Medicine, University of Naples, Italy.

DÉSULFURATION DU CHARBON PAR PROCÉDÉ BIOHYDROMÉTALLURGIQUE: RAPPORT D'AVANCEMENT D'UN PROJET DE TROIS ANS

M. Angus(1), *G. Conti*(2), *N. Orsi*(2), *V. Pietropaolo*(2), *G. Rossi*(3),
P. Tataseo(2), *P. Trois*(3) *et P. Valenti*(4)

RÉSUMÉ

Une très grande partie des réserves mondiales de charbon exploitables présentent une teneur en soufre dépassant largement les limites établies dans la réglementation sur la protection de l'environnement de la plupart des pays. La mise au point de procédés efficaces d'extraction du soufre contenu dans le charbon pourrait donc accroître de façon considérable les ressources énergétiques. Le présent article est un compte rendu d'avancement du programme de recherche mis en oeuvre par une équipe multidisciplinaire de chercheurs italiens. Le programme porte sur quatre types de charbons européens dont la teneur totale en soufre est comprise entre 1.6 et 6%, avec proportions variables de soufre organique. On peut extraire jusqu'à 90% du soufre pyritique par l'entremise d'éléments bactériens, à condition que le charbon soit suffisamment pulvérisé et que les constituants de l'agent en suspension soient compatibles avec la composition chimique du charbon. Il est plus facile de biodépyritiser les charbons hautement poreux.

Les souches microbiennes isolées des cultures enrichies et obtenues par échantillonnage répété dans une mine souterraine de charbon sous-bitumineux en Italie, ont été identifiées comme des espèces des genres *Bacillus, Enterobacter, Micrococcus* et *Pseudomonas.*

L'aptitude des souches *Pseudomonas* à décomposer les composés soufrés a fait l'objet d'une analyse au moyen de dibenzothiofène (DBT) utilisé comme molécule de référence. Ce composé a été ajouté au milieu minimum comme seule source de soufre, en plus du benzoate de sodium (NaB) comme seule source de carbone (charbon). Certaines souches sont capables d'extraire plus de 40% du soufre organique et on a pu prouver, par des essais auxanographiques, que le produit de la décomposition finale était constitué par des ions de sulfate et que l'oxydation se produisait par contact direct entre la paroi de la cellule du micro-organisme et le composé chimique. Les souches adaptées ont aussi fait l'objet d'analyse quant à l'activité d'extraction du soufre organique d'un charbon italien et les résultats ont démontré que ces souches pouvaient extraire jusqu'à 18% du soufre organique.

Compte tenu des constatations faites, une fiche de travail a été proposée en vue de la mise en oeuvre d'une usine pilote pour la désulfuration microbienne du charbon.

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(1) Conseil National Italien de Recherche
(2) Institut de Microbiologie, Université "La Sapienza", Rome, Italie
(3) Département des Mines et du Traitement des Minéraux, Université de Cagliari, Italie
(4) Institut de Microbiologie, 1ère Faculté de médecine, Université de Naples, Italie.

INTRODUCTION

The steady growth in universal energy demands, unlikely to change in the near future, raises the problem of the correct and complete utilization of all available energy sources. Of these, coal occupies a very prominent position, since it comprises about 75% of the world's total fossil fuel resources (Klein *et al.*, 1988). Indeed, coal reserves mineable with current mining techniques were estimated back in 1974 at more than 1000 x 10^9 Mg (Fettweiss, 1976), while present world coal consumption should not exceed 4.5 x 10^9 Mg per year.

A considerable proportion of this amount is burned for generating electricity: 2.5 x 10^9 Mg CE (CE = coal equivalent; 1 Kg CE = 29.3 x 10^3 KJ) in 1985 in the western world alone for producing 7000 TWh of electricity (Klein *et al.*, 1988).

The total sulfur contents of coal may, however, represent an insurmountable obstacle to its utilization since, according to the environmental regulations presently enforced in most industrialized countries, a coal is considered "high sulfur" and cannot be burned as received, when its total sulfur content exceeds 1%. Although no reliable statistics are available on the proportion of high-sulfur coals over total coal reserves, the undisputed occurrence of acid depositions that are causing so much concern over the destruction of vegetation in Europe and North America, is indicative of the extent of the problem. Table 1, which gives some examples of high-sulfur coals together with their polluting potential in terms of grams SO_2 per MJ electricity generated, shows that such coals are found in many countries.

For certain countries, such as Italy, France and Spain, whose mineable coal reserves are small compared to other industrialized countries and to their energy needs, the exploitation of high-sulfur coals in their coal basins plays an important economic role.

A profitable process of removing sulfur from coal might therefore represent a major step toward a rational and environmentally safe utilization of coal resource.

For this reason, some years ago the Mining and Mineral Dressing Department (MMDD) of the University of Cagliari drew up a research program aimed at investigating various processes of sulfur removal from coal, including the possibilities offered by microbiological methods.

The validity of the program was recognized by the Commission of European Communities (in the framework of a project involving Bergbau Forschung GmbH of Essen in Germany, Biotechnology Delft in the Netherlands and Warren Spring Laboratories in the UK) and by Enichem-Anic, who granted financial support. Owing to the broad scope of the research and to the need for the cooperation of experts of various disciplines, the MMDD invited researchers of the Universities of Rome and Naples to join the project.

Both pyritic and organic sulfur removal by microbiological methods were considered in the program. This paper reports on the results obtained in three years of activity.

The research program was started by the Italian group on September 1st 1986 and will terminate at the end of 1989.

It should be pointed out that, to the best of the authors' knowledge, no direct and reliable method of organic sulfur analysis exists nor is it possible to identify with certainty the types and amounts of the various hydrocarbons contained in the coal matrix to which sulfur is bound. These are two serious drawbacks. Actually the quantitative determination of organic sulfur as the difference between total sulfur and the sum or pyritic, sulfate and elemental sulfur, is not very reliable, especially in view of the uncertainties in elemental sulfur analysis and as a result the efficiency of organosulfur removal cannot be reliably assessed. The uncertainty as to the chemical nature of organosulfur compounds does not allow the selection of microorganisms with the most suitable metabolic pathway.

Consequently, the approach adopted in implementing the research has been empirical and is oriented, for organosulfur removal investigations, towards those model compounds claimed to be present in most coal matrices alongside the coals selected for the investigation.

Coal biodesulfurization is a two-sided problem, at least in the light of and within the limits of present knowledge. Although it has been claimed that some thermophilic and extreme thermophilic microorganisms are capable of removing both pyritic and organic sulfur from coal (Kargi and Robinson, 1982 a,b: Gõckay and Yurteri, 1983), these results do not appear to have been subsequently borne out. Hence in the research program concerned, two different approaches, employing different microbial strains to the bioremoval of the two sulfur types were followed.

In recent years increasing attention has been devoted to organic sulfur bioremoval as the numerous publications testify, and at least two research approaches have emerged: i) the isolation of naturally occurring microorganisms capable of removing sulfur from coal or from model compounds by enrichment culture techniques (Klubek and Clark, 1987; Maka *et al.*, 1987; Agus *et al.*, 1988, van Afferden *et al.*, 1988); ii) the induced mutation of microorganisms with genetic engineering techniques in order to obtain strains displaying increased specificity towards model compounds like dibenzothiophene (DBT) and decreased activity against aromatic rings lacking sulfur (very likely the building blocks of the coal matrix) (Isbister and Kobylinsky, 1985, Klubek and Clark, 1987). This paper reports on the results achieved following the first approach.

MATERIALS AND METHODS

The Investigation Coals

In agreement with the three partners of the CEC project, four coal types were investigated: Sulcis coal, from the Sulcis coal basin in southwestern Sardinia; a mixture of washed coals referred to as "Monopol" coal from the coal beneficiation plant of the Monopol mine in the Ruhr basin (Germany); Gardanne coal, from the Bassin d'Arc in southern France, where the Gardanne mine is operated by the Houillières de Provence; Kellingley coal, from the Kellingley coal mine in the United Kingdom. Tables 2 to 5 summarize the most salient characteristics of these coals. All coal samples were dry ground to 95% by weight passing the 40-pm square mesh screen.

Sulcis coal. The 4-Mg sample was taken from a face in the so-called 1st Seam of the Seruci Mine. It is a sub-bituminous coal or "Glanzbraunkohle" according to ASTM and DIN classifications respectively. Three types of syngenetic pyrite occur in Sulcis coal: framboidal pyrite, pyrite belonging to the euhedral class (Figure 1) and massive aggregates. Owing to the very fine dissemination of pyrite, physical methods of pyritic sulfur removal have proven unsuccessful until now. A feature of the investigated sample is the constant presence of carbonatic phases (dolomite and calcite) filling the cracks and fissures of the coal phase (Figure 2), predominantly inside the vitrinite group. Pyritic sulfur is always accompanied by higher proportions of organic sulfur. More details on Sulcis basin and coal can be found in the papers by Agus and Carta (1976), Carta *et al.*, 1978, Agus (1981) and Agus *et al.*, 1989.

Gardanne coal. Tests were performed on batches taken from a 700-Kg sample kindly provided by the Houillères de Provence. This is also a sub-bituminous coal, characterized by abundant pyrite and carbonate phases (Figure 3) accompanied by quartz and clay. Pyrite, basically syngenetic in nature, is finely dispersed throughout the coal matrix in the form of individual crystals (sometimes submicron in size) and of framboidal clusters ranging from 5 to 40 micrometers (Figure 4). Organic sulfur is always present in high percentages. More details on this coal, which is genetically and petrographically very similar to the Sulcis coal and is presently burned in thermoelectric power stations, can be found in the papers by Boudou *et al.*, 1978, Carta (1979), Agus and Carta 1982, and Agus 1988.

Monopol coal. This is a bituminous coal in which pyrite occurs in both the syngenetic and epigenetic forms. The syngenetic pyrite appears as individual framboids ranging from a few up to 40 micrometers, with a predominance of the 20 micrometer size, and less frequently as groups of framboids. Epigenetic pyrite is present in the form of fissure fillings (Figure 5) and is frequently accompanied by marcasite. The veins of epigenetic pyrite range in thickness from 20 to 300 micrometers, with 100 micrometers prevailing. Pyrite is also present in cellular cavities of semifusinite and fusinite structures. Of the carbonates, calcite and, to a lesser extent, siderite can be mentioned.

Kellingley coal. On the grounds of "rank" determinations based on the average reflecting power of virtinite, Kellingley coal can be defined as a "high volatile bituminous coal" according to the ASTM classification, or as a "Gassflammkohle" according to the DIN classification. Pyrite is basically syngenetic, occurring as individual crystals from 1 to 3 micrometers in size, less frequently in associations. Framboids are also observed, ranging from 5 to 25 micrometers (Figure 6) and clusters of framboids 200 micrometers in size may also occur.

Culture Media

For cultures of acidophilic microflora and depyritization tests, Silvermann and Lundgren's (1959) 9K medium and variants thereof were used. For enrichment of microbial flora in coal mine environment a Brain Heart Infusion (BHI), the minimal medium M9 (Table 6) (Maniatis *et al.*, 1983) and M9 medium supplemented with 1% DBT were used. The culture media were Luria broth (LB), McConkey's medium, M9 + 1.5% Sodium benzoate, Isbister's medium MM (Couch, 1983). Specific solid culture media for the different bacterial growth were: blood agar for *Bacillus*; McClonkey's medium for *Enterobacter*; enterococcosel for *Enterococcus* and LB for *Pseudomonas*. For strain maintenance, M9 medium supplemented with 1% DBT was used; for auxanographic tests Agarose/M9 solid medium. Variants of the above media were used from time to time. 0.1% DBT solutions, prepared from 10% stock solutions in dimethylformamide (DMF) were added to the culture media. Given the low solubility of DBT in the media, it was added in three different ways: directly at room temperature, by heating and vigorously stirring, by sonication.

The Microbial Strains

A distinction has to be made between pyritic and organic sulfur removal. For pyrite removal only the well-known chemolithotrophic acidophiles of the genus *Thiobacillus* were used. Work is still under way to isolate or test microorganisms that exhibit a certain ability to remove organic sulfur.

Pyritic sulfur removal. The "FC" strain, isolated some years ago from the acid drippings of the Fenice Capanne underground mine (Rossi, 1971) in Tuscany and routinely maintained in Cagliari's laboratory in Silvermann and Lundgren 9K medium, was used.

Organosulfur removal. The high organosulfur removal ability claimed for the microorganism denoted CB1, listed as ATCC No. 39381 and obtained by chemical mutagenesis of a *Pseudomonas* sp. wild strain (Isbister and Kobylinsky, 1985), was not borne out by our tests. It was therefore decided to attempt to isolate "ad hoc" microorganisms either from the microflora present in a model mining environment, or from the microflora adapted to the coal-waste dumps of an operating mine.

Slightly different microflora sampling procedures were developed, depending on the collection site. For isolation underground the set-up shown in Figure 7 was adopted, basically consisting of a 20 dm^3 Mariotte carboy, containing the suitable culture medium, placed on a platform supported by the steel arch of a drift located at 250 meters below sea level, where the temperature was 35°C. The medium was either allowed to spread and percolate over the coal exposed on the drift wall or to flow along a gently slopping groove carved therein, so that the residence time of the medium in contact with the coal was sufficiently long. For sampling from drill-core material recovered at various depths or from the surface of coal-waste stockpiles, the following procedure was developed: small heaps ("miniheaps") were formed with about 10 Kg of this material, in plastic pans of suitable size, with gently slopping bottom. The medium was sprayed on top of the heaps and circulated for several days, and sometimes weeks, by means of peristaltic pumps operating in closed circuit. The medium was made up daily to compensate for evaporation, and a few cubic centimeters were periodically sampled and used for isolation of microorganisms.

Sampling from run-of-mine coal was carried out in devices similar to the leach columns currently used for biohydrometallurgical tests. Each column, of PVC pipe 1.70 m in length and 190 mm inner diameter, terminates at its upper end with a cylindrical medium-spraying plexiglass device and at its lower end with a coal-supporting grid and a plexiglass collecting cylinder. The column is filled with coal of the desired size, and the medium is circulated through it by means of a peristaltic pump. The column is air-tight and suitable vents are provided for air inflow and outflowing air sampling.

Several tens of pure strains have been isolated so far from samplings done with both complete and minimal media as well as minimal media supplemented with selective agents. All of these strains are being systematically tested for their ability to attack organosulfur either in model compounds or in coal, and up to now the following strains have been isolated:

- from complete medium *Bacillus* sp., *Enterobacter* sp., and *Micrococcus* sp.:
- from minimal medium, and minimal medium supplemented with dibenzothiophene (DBT) as sole carbon source, several Gram-positive and Gram-negative bacteria, with a prevalence of strains belonging to *Bacillus* and *Pseudomonas* genera.

Furthermore, the liquid medium recovered after percolation along the coal surface exposed on the drift wall was used as inoculum on the following media: LB, McConkey's, M9 + 2% (w/v) glucose; M9 + 1.5% (w/v) sodium benzoate, NaB; M9 + 1.5% (w/v) NaB + 1% (w/v) DBT; M9 + 1% (w/v) DBT; MMA + 5% (w/v) coal; M9 + 5% (w/v) coal. Only the colonies able to grow on (M9 + NaB) medium or on (M9 + NaB + DBT) media were selected and subsequently identified. The genera identified are listed in Table 9.

Pseudomonos putida PAW 340 (strain kindly supplied by Dr. Zennaro of the University of Rome) and Isbister's strain CB1 were used as controls. These strains underwent the same subculturing as those under investigation in order to reproduce the same selection procedure.

For auxonographic tests, either *Escherichia coli* RIV strain or *Pseudonomas* IH (see Table 9), which grows more homogeneously than the former on solid media, were used.

Reagents

All reagents used in the tests were analytical grade. DBT sulfone was supplied by Aldrich, DBT by Fluka A.G., Bruchs, Switzerland, dimethylformamide by Merck, Darmstadt, Germany and magnesium sulfate and ethylenediaminotetraacetic acid (EDTA) by Carlo Erba, Milan, Italy.

Leaching Techniques

All the sulfur removal tests were conducted in 250 or 300 mL Erlenmeyer flasks incubated on gyratory shakers.

Depyritization tests. For these tests, the flask bottoms were fitted with baffles and a New Brunswick type G24 environmental incubator shaker was operated at 26.16 rad.s^{-2} and 28.5°C. Carbon dioxide was supplied to the shaker during all the tests, so as to keep its proportion in the shaker atmosphere above 1%.

Organosulfur removal tests. The tests were carried out in 300 cm^3 flasks containing either organosulfur compounds presumably present in the investigated coals (e.g. DBT) or coal (5% w/v) along with the suitable culture medium.

Analytical Method

Total sulfur was determined by means of the SC132 Instrument manufactured by LECO (St. Joseph, MI, USA). Pyritic sulfur was determined by gravimetric analysis, whereas iron and other elements were analysed by complexometric titration.

Organic sulfur was determined as the difference between total sulfur and pyritic, elemental and sulfate sulfur. The analysis of the residual DBT after microbial attack was done using an original method developed by the Industrial Chemistry Department of Bologna University (Pifferi, 1988).

Hydrogen ion concentrations were determined by means of potentiometric pH-meters of various makes (Beckmann, Orion and Hanna) and redox potentials with a Mod. H1 8418 Hanna electronic potentionmeter with Pt combined electrode.

When required, ferrous and ferric iron concentrations of the leach liquors were determined by the α - α' dipyridil method, using a Bausch & Lomb Spectronic 20 spectrometer.

Auxanographic Tests

In order to ascertain the ability of the microorganisms used in the investigations to degrade DBT and to identify the nature of the degradation products, auxanographic tests were performed. For this purpose, a microbial strain (the "test strain") inhibited by DBT and its degradation products (e.g., DBS) but able to utilize sulfate (SO_4) as sole sulfur source for growth, was used. This strain was preliminarily cultivated in M9 minimal medium, from which sulfur was absent, then harvested to obtain a pellet by centrifugation. The microbial suspension, obtained after two washings of the pellet in saline solution, was then sprayed over an Agarose-M9 solid medium. On the surface of the plates prepared in this way, blank discs (Oxoid) were placed, saturated with samples of the DBT-containing media, where the microbial strains assumed to degrade DBT were grown. The plates were finally incubated for several days at 37°C. The test strain grew only on those discs where SO_4 was present.

Biochemical Tests

In order to identify the location of the solubilized colloidal sulfur and its oxidized forms in the cell, a cell-staining method has been developed. This method consists of inoculating the strain being examined in M9 + 0.1% DBT + 1.5% NaB and incubating it at 30°C and 21 rad.s^{-1}. As negative control a known strain, e.g. *Escherichia coli,* is used. As soon as the number of cells per cubic centimeter reaches 10^7 to 10^8, the culture is centrifuged at 524 rad.s^{-1} for 30 minutes at 4°C. The pellet thus obtained is washed twice with sterile water. To the microbial suspension thus formed, 2.5% $AgNO_3$ solution is added and incubated for 30 minutes. Hydroquinone (0.25 of volume) is then added to the suspension which is incubated for another 2 minutes.

The pellet obtained by centrifiguration at 942 rad.s^{-1} for 15 minutes is washed once again with sterile water and finally centrifuged for a further 15 minutes at 942 rad.s^{-1}. The pellet material is then examined under the electron microscope.

Microbial Identification and Growth-Measurement Procedures

The microbial strains were identified according to the standard procedure reported in Bergey's Manual (1984).

Cell concentrations were obtained by the dilution plating method and by counting colony-forming units (CFU) per cm^3.

RESULTS

Pyrite Sulfur Removal

Test were carried out on the four coal types available. The parameter "E" from equation (1):

$$E = 1 - \frac{S_{pyr}}{(S_o)_{pyr}} \tag{1}$$

where: E = metallic sulfide sulfur solubilization effectiveness;

S_{pyr} = metallic sulfide sulfur assay in desulfurized coal;

$(S_o)_{pyr}$ = metallic sulfide sulfur assay in run-of-mine coal

was adopted as a measure of metallic sulfide sulfur ("pyritic sulfur") solubilization effectiveness.

Operating conditions being equal, the achievement of optimum effectiveness for every coal type depends on culture medium composition. Actually, as can be seen from Tables 7 and 8, which summarize the results of an ANOVA experimental program, the salts of the culture medium are the same as those of Silvermann and Lundgren's but their proportions vary considerably from one coal to another (Rossi and Trois, 1989).

Standard test conditions were: 250-cm^3 baffled-bottom Erlenmeyer flasks containing 92 g of medium, 5 g of inoculum and 3 g of pulverized coal; shaker speed, 26.16 rad.s^{-1}; temperature, 28°C.

In order of biodepyritization effectiveness, the Sulcis coal appears to be the most amenable followed by Gardanne, whereas Monopol and Kellingley appear to be the most recalcitrant. No appreciable pH variations were observed whereas the Eh always rose above 630 mV.

Organic Sulfur Removal

The strains shown in Table 9 were subcultured by means of serial passages in solid M9 medium supplemented with NaB-DBT at the indicated concentrations. The growth in liquid M9 medium supplemented with NaB+ DBT yielded positive results for all of the examined strains and always attained the number of 5.10^7 cells per cm^3. Only the strains preselected in the mine using basal salt solution plus DBT exhibited significant growth when cultivated in the presence of this selective agent. Table 10 shows the percent DBT degradation yield by the strains inoculated in M9 + DBT.

A number of strains were observed to grow on the same solid medium (M9) in the presence of DBT as sole carbon source, i.e. without NaB, where strain CB1 growth is partially inhibited. The same strains were inoculated into Erlenmeyer flasks containing 50 cm^3 of M9 medium and 2.5 g of pulverized Sulcis coal and one of them produced more than 15% organic sulfur removal. Under the above experimental conditions, microbial growth customarily attains from 3.10^7 to 5.10^7 cells per cm^3.

Another series of tests was carried out using 1% (w/v) DBT as sole carbon and sulfur source. The data reported in Table 11 concern the results obtained with the strains adapted and tested up to the time of writing. The highest microbial degradation is achieved when DBT is solubilized in the culture medium by sonication or by heating.

Better degradation was also obtained when mixed cultures were used. When the strains exhibiting the highest DBT-degradation potential (Tables 10 and 11) were cultivated together, 30% and 42% DBT degradation were observed after 72 and 360 hours incubation (at room temperature and 20.9 $rad.s^{-1}$) respectively.

As for coal-degrading activity, removal of between 10 and 18% organic sulfur was observed. Under these experimental conditions, no degrading activity was detected for the CB1 strain.

With a view to elucidating the sulfur-oxidation mechanism of the isolated strains, the liquid growth medium was supplemented with EDTA, so as to achieve a 100-mM concentration. The assays for residual sulfur showed that microbial DBT utilization increased by about 10% in the presence of EDTA compared to control cultures, owing to enhanced cell-wall permeability.

Auxanographic tests. The samples of media from various bacterial cultures (M9 + 0.1% DBT and 0.15% NaB) at different incubation time indicated the biological degradation of DBT with SO_4 formation. Of the strains of Tables 10 and 11, IS strain seems to be the most effective in DBT biodegradation (19%).

Biochemical tests. The $AgNO_3$ staining method showed the presence of black dots in the inner space of the 1S-strain cells.

Finally, it has been ascertained that the sulfur-removal activity from DBT of the 1S-strain is enhanced by about 4% in the presence of iron.

CONCLUSIONS

Pyrite can be satisfactorily removed from the four coals investigated by means of *Thiobacillus ferrooxidans*. However the process is affected to a large extent by certain factors which have to be taken into due account if optimum operating conditions are to be achieved.

One such factor is the chemical composition of the run-of-mine coal, particularly phosphorus and probably other elements or compounds, which calls for suitable culture medium ingredients. This is in agreement with the findings and recommendations of Hoffmann *et al.* (1981) and Kos *et al.* (1983). Porosity also seems to be an important factor, since it may considerably affect the accessibility of pyrite to microorganisms or, at least, to ferric ions. Sulcis coal, where the pyrite is as finely – if not more so – disseminated as in Kellingley and Monopol coals, appears to be more readily amenable to depyritization and, as can be seen from Table 7, it has much greater porosity than the other two coals.

One major drawback of current process technology is, however, the limit of 20% solids concentration in the pulp, beyond which pyrite bioremoval effectiveness declines. This limit involves high reactor volumes and makes the process less competitive. Identification of the causes of this limitation and overcoming it would contribute decisively towards the commercial application of pyrite bioremoval.

As far as organic sulfur removal is concerned, the repeated collection of microflora samples has led to the isolation of several microbial strains capable of removing organic sulfur from both model compounds and coal. The most promising results were obtained with various *Pseudomonas* strains, which displayed an ability to remove more than 40% S from DBT and up to 18% S from Sulcis coal. These results lend weight to the findings of other authors (Klubek and Clark, 1987; Agus *et al.*, 1988; Yamada *et al.*, 1968).

Auxanographic analysis revealed that these microorganisms oxidize organic sulfur to sulfate, water-soluble ionic species that can be easily removed. Furthermore, organic sulfur oxidation requires the contact of the microbial cell wall with the single sulfur atoms, as shown by the enhanced sulfur removal observed when the culture medium was supplemented with EDTA. The $AgNO_3$ test seems to provide evidence that the examined strain utilizes the sulfur oxidation products derived from DBT degradation as a sulfur source. Hence, the practically uniform dispersion of sulfur atoms throughout the coal matrix makes their complete removal impossible, since only those exposed on the outer surface of the grains or on the large pore surfaces can be reached by the microorganisms. The larger the surface-to-volume ratio, the more the sulfur is amenable to microbial attack. However, the surface-to-volume ratio cannot be increased beyond a certain value, owing to the high grinding costs and to the depreciation of coal with grain size smaller than market specifications. For the time being, the finest coal particles may find commercial application in the coal-water mixtures technique, with an average grain size larger than 40 micrometers (Henderson, 1981). It is therefore imperative to resort to other means of increasing the exposed surface. One possibility is the preliminary depyritization and dissolution of the carbonate phases present in coal (Figure 8). The surfaces of all the cavities left by pyrite and carbonate removal contribute to coal matrix, and hence organic sulfur, exposure. The flowsheet of the sulfur bioremoval plant shown in Figure 9 was developed (Orsi *et al.*, 1989) with these considerations in mind.

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Table 1

Examples of Average Sulfur, Ash and Heating Values of some Coal Samples

Country/State	County	Coal	kJ/Kg	% Ash	% Total Sulfur	g SO_2 per MJ
France		Gardanne	28,360	10.84	5.67	4.0
Italy	Sulcis	I Seam	20,485	7.7	5.9	5.75
Spain		Berga	15,900	41.0	4.75	6.0
		Utrillas	21,850	18.6	7.92	7.2
USA						
Ohio	Belmont	Pittsburg	28,600	15.1	6.19	4.3
	Coshocton	Middle Kittanning	28,360	14.1	6.39	4.5
	Muskingum	Lower Kittanning	28,800	11.5	4.85	3.4
Pennsylvania	Indiana	Lower Kittanning	31,380	12.9	4.68	3.0
Utah	Emery	Blind Canyon	31,850	5.6	0.58	0.3
Virginia	Wise	Taggart	33,940	2.9	0.79	0.5
W. Virginia	Preston	Upper Freeport	28,800	18.5	2.44	1.7
	Nicholas	Middle Kittanning	27,900	20.2	1.06	0.8
Wyoming	Sweetwater	Rock Spring No. 11	29,060	6.5	0.97	0.7

Table 2

Chemico-technological characteristics of Sulcis coal (Sardinia)

Component	Mass %
Ash (including oxides	7.7
Total sulfur	5.9
Pyritic sulfur	1.06
Sulfate sulfur	0.8
Total iron	1.4
Cao	1.5785
K_2O	n.d.
MgO	0.8624
P_2O_5	0.0616
Porosity %	31.46

Table 3

Chemico-technological characteristics of Arc coal (France)

Component	Mass %
Ash (including oxides	7.5
Total sulfur	5.1
Pyritic sulfur	1.06
Sulfate sulfur	0.21
Total iron	0.7
CaO	1.50
K_2O	0.0225
MgO	0.255
P_2O_5	0.1725
Porosity %	29.09

Table 4

Chemico-technological characteristics of Ruhr coal (German Federal Republic)

Component	Mass %
Ash (including oxides	28.5
Total sulfur	1.7
Pyritic sulfur	1.33
Sulfate sulfur	0.21
Total iron	2.6
K_2O	1.254
Na_2O	0.3135
MgO	0.5415
P_2O_5	0.1425
Porosity %	4.5

Table 5

Chemico-technological characteristics of Kellingley Mine coal (United Kingdom)

Component	Mass %
Ash (including oxides	9.1
Total sulfur	1.77
Pyritic sulfur	1.07
Sulfate sulfur	0.12
Fe_2O_3	1.2558
CaO	0.2275
MgO	0.1274
Na_2O	0.1001
P_2O_5	0.0637
K_2O	0.0546
Porosity %	19.4

Table 6

Composition of M9 culture medium

Chemical Compound	Amount g dm^{-3}
KH_2PO_4	3.0
Na_2HPO_4	6.0
NaCl	0.5
NH_4Cl	1.0

Table 7

Optimum conditions for biodepyritization of some coals

Coal type	Pyritic sulfur %	Porosity %	Medium (*)	Initial pH	Final pH	Initial Eh (vs.SCE) mV	Final Eh (vs.SCE) mV	Pyrite removal %
Gardanne	1.06	29.09	M_1	2.23	2.27	391.5	637	0.86
Kellingley	1.07	19.04	M_2	2.15	2.22	449	633	0.69
Monopol	1.33	4.5	M_3	2.04	2.03	512	672	0.69
Sulcis	1.06	31.46	M_4	2.23	2.03	357.3	635	0.92

(*) Media compositions are shown in Table 8.

Table 8

Compositions of optimal media for coals tested.

Salt	Media Composition ($g\ dm^{-3}$)			
	M_1	M_2	M_3	M_4
Ammonium sulfate	3	1.5	0	0.75
Potassium chloride	0.025	0	0.1	0
Potassium dihydrogen phosphate	0.125	0.5	0	0.25
Mg sulfate	0.5	0.5	0.5	0.5
Ca nitrate	0.015	0.015	0.015	0.015

Table 9

Strains selected and identified from enrichment cultures collected in Seruci Mine (Sardinia)

Strain	Genus
1A	*Pseudomonas putida*
1B	*Pseudomonas putida*
1H	*Pseudomonas putrefaciens*
1L	*Bacillus* ssp.
1M	*Bacillus* ssp.
1N	*Bacillus* ssp.
1P	*Pseudomonas fluorescens*
1Q	*Pseudomonas putida*
1R	*Bacillus* ssp.
1S	*Xantomonas*

Table 10

% DBT degradation by single strains of Table 9

Strain	% DBT	Degradation
	72 hours	360 hours
1A	0	12
1B	0	10
1L	0	0
1M	0	13
1N	0	11
2P	0	18
1Q	2	14
1R	0	0
1S	0	19
PP	0	14
CB1	0	19

Table 11

% DBT degradation by adapted strains of Table 9

Strain	% DBT degradation
1H	31
1M	12
1N	31
2P	33
1Q	44
1R	44
PP	44

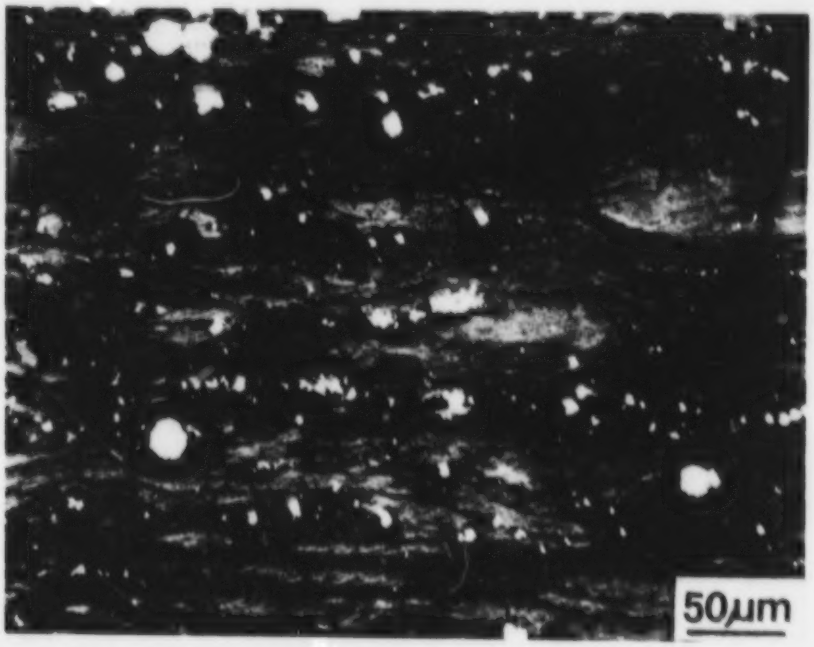

Fig. 1 Sulcis coal: finely dispersed individual crystals and framboids of pyrite. Polished surface. Oil imm.

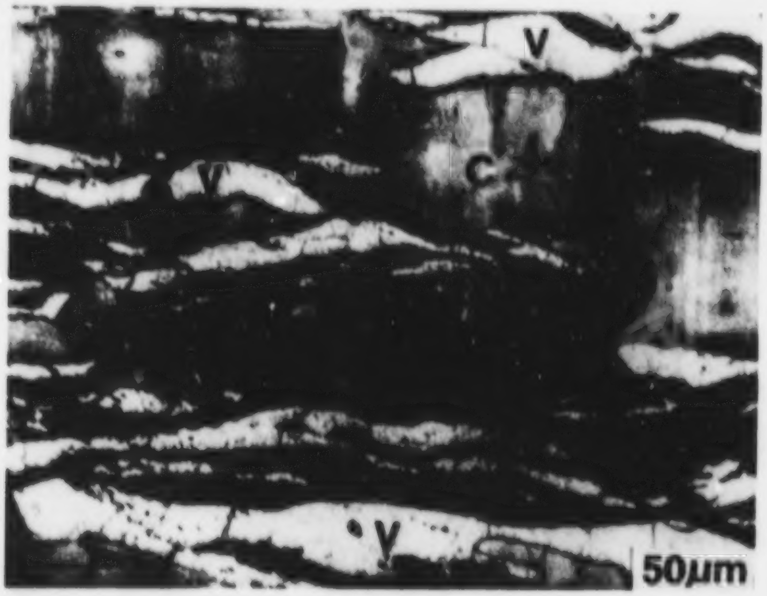

Fig. 2 Sulcis coal: carbonatic phase (c) in fissures and cracks in vitrinite (v). Polished surface. Oil imm.

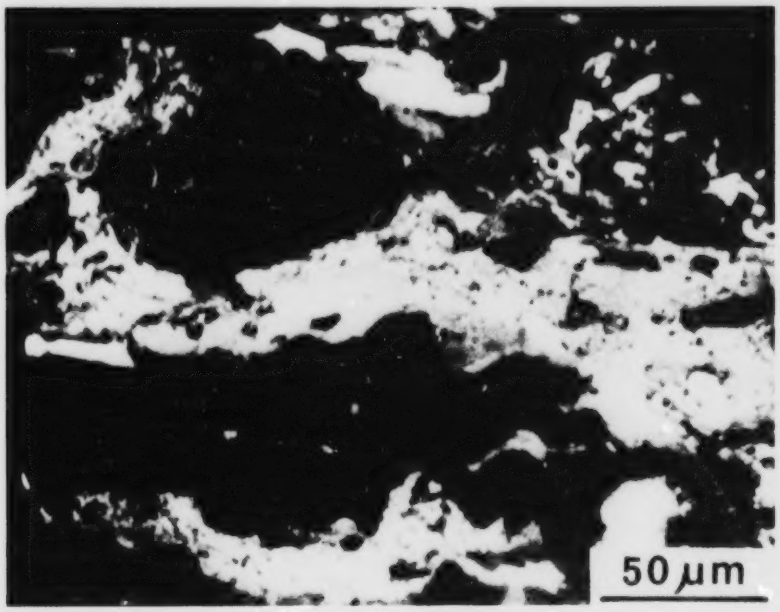

Fig. 3 Gardanne coal: carbonatic elements (dark) disperses within the coal matrix. Polished surface. Oil imm.

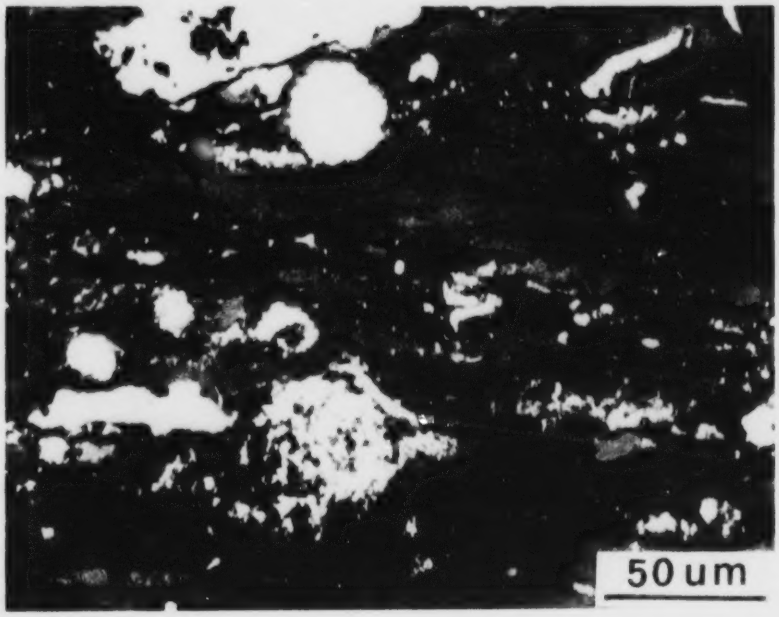

Fig. 4 Gardanne coal: framboids and individual pyrite crystals. Polished surface. Oil imm.

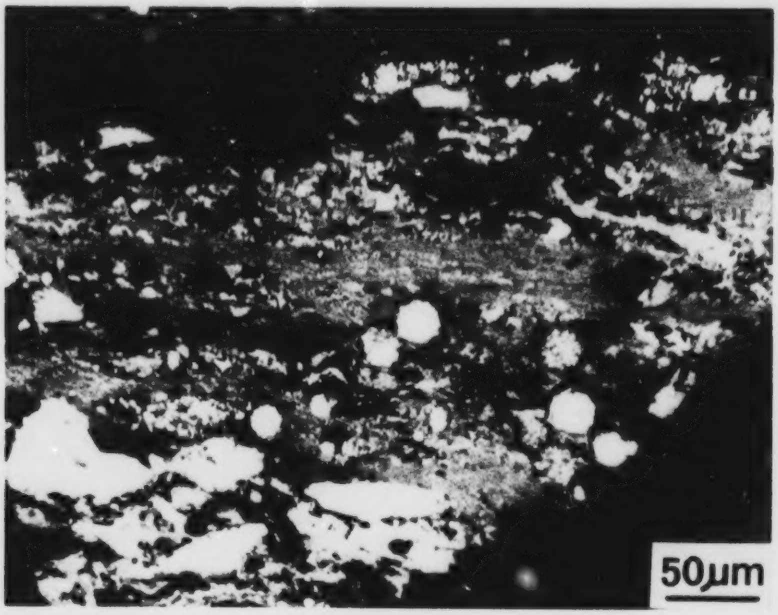

Fig. 5 Kellingley coal: fine dispersion of pyrite framboids and individual crystals. Polished surface. Oil imm.

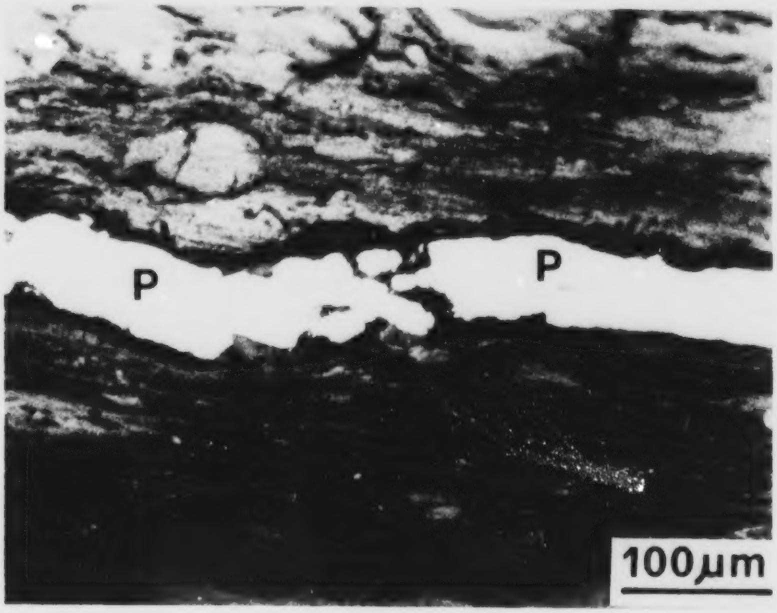

Fig. 6 Monopol coal: epigenetic pyrite (P) inside fissures of coal matrix. Polished surface. Oil imm.

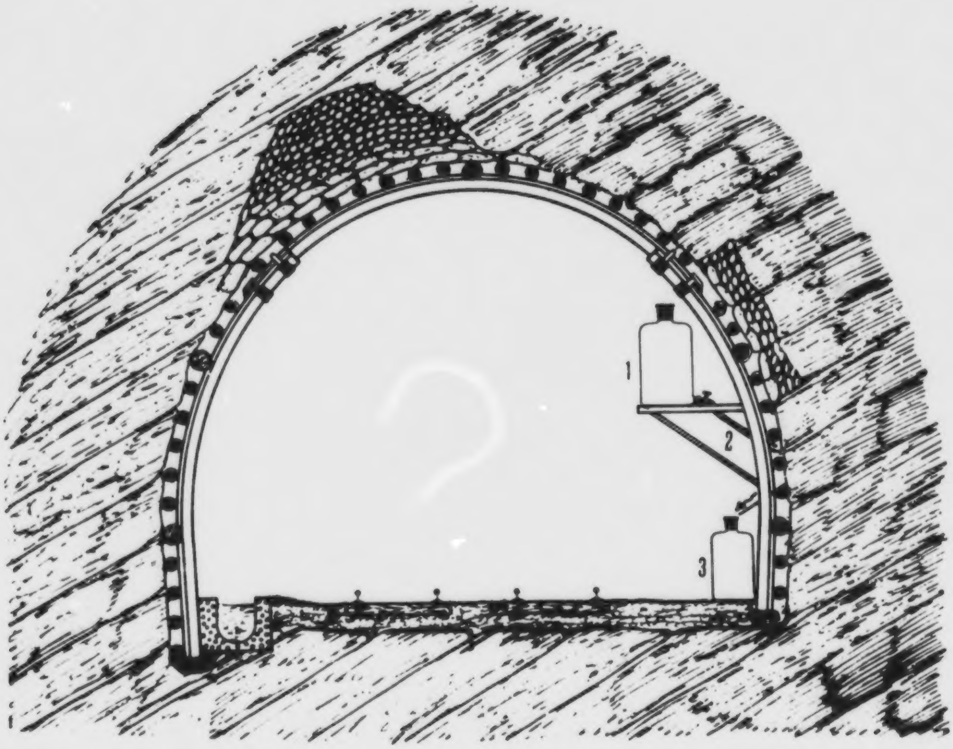

Fig. 7 Set-up for collecting microflora in the Seruci mine. 1 - head tank; 2 - pipe; 3 - medium collecting carboy.

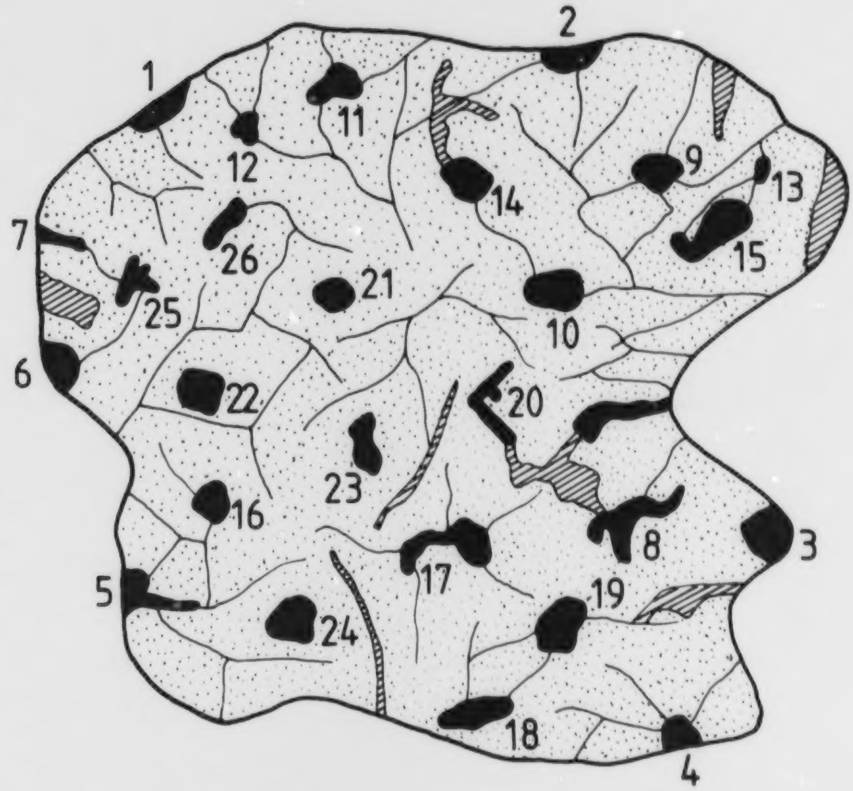
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Fig. 8 Two-dimensional model of coal particle. Black grains represent pyrite; dashed grains, carbonates; white matrix, coal; lines are pores or cracks.

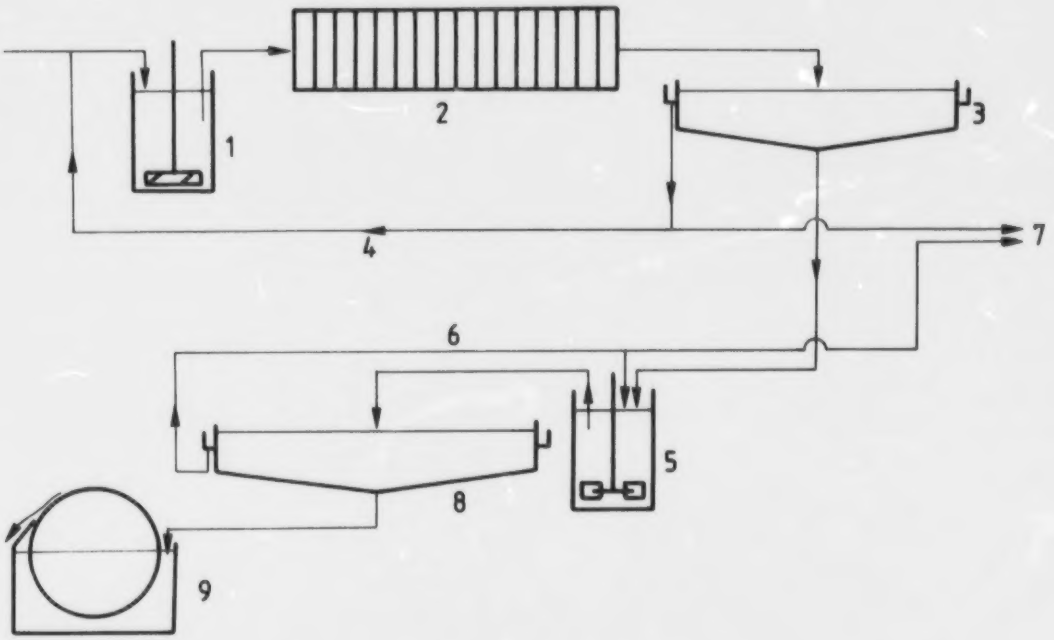

Fig. 9 Flowsheet of a microbial coal desulfurization pilot plant. 1 – backmix reactor as microbial propagation; 2 – plus flow reactor for pyrite removal; 3 – thickener; 4 – supernatant recycle; 5 – reactor system for organic sulfur removal; 6 – supernatant to tailings pond; 7 – tailings pond; 8 – thickener; 9 – filter;

THE ROLE OF GROWTH AND MAINTENANCE IN THE OXIDATION OF PYRITE IN BATCH CULTURE BY A MODERATELY THERMOPHILIC, FACULTATIVE CHEMOLITHOAUTOTROPH.

F.C. Boogerd, M.M.Q van Alphen, W.J. van Anrooij, J.C. de Bruyn, P. Bos and J.G. Kuenen

Department of Microbiology and Enzymology
Kluyver Laboratory of Biotechnology Julianalaan 67 2628 BC Delft the Netherlands

ABSTRACT

An acidophilic, moderately thermophilic bacterium was isolated from the indigenous population of high-pyritic coal. The rod-shaped isolate had a temperature optimum of 45°C and a pH optimum of 1.5. It was able to grow heterotrophically on yeast-extract. The addition of pyrite to media, containing yeast-extract, resulted in an increased maximum number of bacteria. However, high concentrations of yeast-extract inhibited bacterial growth. In the presence of 0.01% yeast-extract and at concentrations of pyrite higher than about 20 mM, the amount of biomass formed was limited by the yeast-extract. Consequently, no first-order dependence on pyrite was found for the rate of pyrite oxidation. In contrast, the bacteria oxidized pyrite at a, more or less, constant rate at the moment the maximal amount of biomass was formed.

Although in the absence of yeast-extract vitually no growth occurred, pyrite was still oxidized at a rate proportional to the inoculum size. An obvious stimulation of the oxidation of pyrite was observed when air was replaced by 5% (v/v) CO_2-enriched air at the moment about half of the initial 73 mM pyrite was oxidized. In a similar experiment with an initial concentration of 15 mM pyrite no such stimulation was found. The rate of pyrite oxidation did neither change when CO_2-enriched air was substituted by air at the moment about 60% of the initial 73 mM pyrite was converted.

All the observations could be accomodated to a model in which the growth of bacteria, the bacterial need for maintenance-energy and the maximum capacity of transfer of pyrite from solid to the liquid phase are important parameters.

RÔLE DE LA CROISSANCE ET DU MAINTIEN D'UNE BACTÉRIE CHIMIOLITHOAUTOTROPHE FACULTATIVE, MODÉRÉMENT THERMOPHILE DANS L'OXYDATION DE LA PYRITE EN CULTURE DISCONTINUE

F.C. Boogerd, M.M.Q. van Alphen, W.J. van Anrooij, J.C. de Bruyn, P. Bos et J.G. Kuenen

Département de Microbiologie et d'Enzymologie
Laboratoire de Biotechnologie Kluyver, Julianalaan, Pays-Bas

RÉSUMÉ

Une bactérie acidophile, modérément thermophile a été isolée dans une population indigène de charbon hautement pyritique. La bactérie en forme de bâtonnet avait une température optimale de 45°C et un pH optimal de 1.5. Elle a pu croître de façon hétérotrophique dans de l'extrait de levure. L'addition de pyrite au milieu contenant de l'extrait de levure a permis d'obtenir une croissance bactérienne maximale. Cependant, les concentrations élevées d'extrait de levure ont inhibé la croissance des bactéries. En présence de 0.01% d'extrait de levure et à des concentrations de pyrite supérieures à 20 mM, la biomasse produite était limitée par l'extrait de levure. Par conséquent, on n'a pas pu établir un facteur limite de premier ordre de la pyrite en fonction de son taux d'oxydation. Par contre, les bactéries ont oxydé la pyrite à un rythme plus ou moins constant, au moment de la formation de la quantité maximale de biomasse.

Bien qu'en absence d'extrait de levure, la croissance était pratiquement inexistante, la pyrite s'oxydait tout de même à une vitesse proportionnelle à la taille de l'inoculum. On a constaté une stimulation évidente de l'oxydation de la pyrite lorsque l'air était remplacé par de l'air enrichi avec 5% de CO_2 (par poids) au moment où environ la moitié de la pyrite initiale (73 mM) était oxydée. Au cours d'une expérience semblable, avec une concentration initiale de pyrite de 15 mM, on n'a pas constaté de stimulation de ce genre. La vitesse d'oxydation de la pyrite restait constante lorsque l'air enrichi en CO_2 était remplacé par de l'air ambiant au moment où environ 60% de la quantité initiale de pyrite 73 mM était transformée.

Toutes les constatations pourraient faire l'objet d'un modèle dans lequel la croissance des bactéries, le besoin en bactéries pour assurer le maintien de l'énergie et la capacité maximale de transfert de la pyrite de l'état solide à l'état liquide, seraient des paramètres importants.

INTRODUCTION

Since the energy crises in the seventies the microbial desulfurization of coal was studied by many research institutes (Bos *et al.*, 1986). Previously, the feasibility of desulfurization of low-pyritic coals by mesophilic, autotrophic bacteria was investigated (Bos *et al.*, 1986; Bos *et al.*, 1988). Simple suspension reactors, such as cascades of Pachuca-tanks, appeared to be appropriate to fulfil the requirements for adequate aeration and bulk mixing, and to prevent sedimentation in the bottom of the reactors. Recently, an investigation into the potential of this process design for the treatment of high-pyritic coals has started. This study is part of an EEC-project on the microbial desulfurization of coal in which laboratories from the United Kingdom (Warren Spring Laboratory), the Federal Republic of Germany (Bergbau Forschung GmbH) and Italy (University of Cagliary) co-operate with our laboratory.

It is a well-known fact that the oxidation of pyrite by oxygen is a highly exothermic reaction. Therefore, the microbial desulfurization of high-pyritic coals results in an increase of the temperature in the reactor such that the growth of, and pyrite oxidation by, mesophilic bacteria would be inhibited if the reactor was not cooled. Since cooling is expensive, in this case thermophilic, pyrite-oxidizing bacteria might be more appropriately used.

Previously, it was emphasized that attention should be devoted to both the kinetics of bacterial growth and the kinetics of pyrite oxidation (Huber *et al.*, 1984; Bos *et al.*, 1988). The overall rate of leaching of pyrite by mesophilic, autotrophic bacteria was reported to exhibit a first-order dependence on the total surface area of FeS_2 (Hoffmann *et al.*, 1981; Andrews and Maczuga, 1984; Huber *et al.*, 1984). This phenomenon was reminiscent of the idea that the amount of biomass formed in batch culture increased in proportion to the initial surface area of pyrite. Autotrophic growth ceased when pyrite became limiting, and after that the oxidation of FeS_2 could be described by first order characteristics. Bos and Kuenen (1989) have calculated the first order constants from several published data on residence time and percentage pyrite removal. Values between 0.9×10^{-3} and 12.0×10^{-3} (1/h) were obtained.

Several acidophilic, moderately thermophilic bacteria were isolated and these bacteria grew optimally in the presence of both an inorganic electron donor, like Fe^{2+}, FeS_2 or reduced sulfur-compounds, and yeast-extract (Brierley and Lockwood, 1977; Brierley *et al.*, 1978; Norris *et al.*, 1980; Hendy, 1987). For some of these bacteria the organic nutrient requirement was resolved into distinct requirements for an organic carbon substrate and for reduced assimilative sulfur in either organic or inorganic form (Norris *et al.*, 1980; Brierley *et al.*, 1978). In the presence of air, Fe^{2+} and yeast-extract virtually none of of the cell-carbon of the isolate TH1 was derived from CO_2 (Brierley *et al.*, 1978). The optimal percentage of yeast-extract was found to be 0.005-0.02% and the growth rate was reduced with lower or higher concentrations (Brierley *et al.*, 1978). Thus, these bacteria showed a chemolithoheterotrophic lifestyle, at least in the presence of air. However, fixation of CO_2 was shown to occur during the oxidation of Fe^{2+} (Marsh and Norris, 1983a; Wood and Kelly, 1983) and mineral sulfides (Marsh and Norris, 1983b) in the presence of CO_2-enriched air. In this paper we describe the growth of our own isolated, moderately thermophilic bacterium and the kinetic features of the oxidation of pure pyrite-particles. All the results can be accomodated to a model in which the growth of bacteria, the bacterial need for maintenance-energy and the maximum capacity of transfer of pyrite from solid to the liquid phase are of crucial importance.

MATERIALS AND METHODS

Media: A standard liquid medium was used in all the experiments, which contained 1.5 mM $(NH_4)_2SO_4$, 1.0 mM $MgSO_4$, 0.5 mM $CaCl_2$ and 2.0 mM KH_2PO_4. The pH was adjusted to 1.5 with 4 N H_2SO_4 or 4 N HCl. The media were sterilised at 120°C for 20 min, after the addition of the desired amount of pyrite. Yeast-Extract (YE; Bacto Yeast-Extract; Difco Laboratories, USA) was added from a filter-sterilised (0.2 μm) concentrated stock solution.

Isolation of the acidophilic, moderately thermophilic bacterium: The indigenous population of high-pyritic [6.5% (w/w) FeS_2] coal from Zambia (MAAMBA-mine; seam A) was obtained as follows: the coal was crushed to particles with a mean diameter of 50 μm. Flasks (250 ml) were filled with 100 ml 5% (w/v) coal-water slurry of pH 1.8, supplemented with 5 mg $(NH_4)_2SO_4$ and 2.5 mg K_2HPO_4, and incubated in reciprocal shaking (150 rpm) machines at 45°C. During the first three days of incubation the pH tended to increase due to the dissolution of alkaline components (e.g., carbonates) from the coal. Therefore, the pH was readjusted to 1.8 when necessary. The loss of water due to evaporation was compensated for by adding acid water. The resulting bacterial population (enrichment culture) was dominated from the start by a rod-shaped bacterium. This bacterium was isolated as a pure culture by means of the "floating filter technique" (J.C. de Bruyn, pers. comm., 1989). The strain was routinely maintained in serum bottles, containing 0.2% (w/v) FeS_2 and 0.01% (w/v) YE in standard medium, which were incubated at 45°C in a reciprocal shaking machine. Every 2-3 weeks the culture was inoculated into fresh medium.

Pyrite and coal particles: The pyrite-particles were 88% pure and the mean diameter of the particles was around 10 μm. Two coal-water slurries (5% w/v) were used, containing coal from the MAAMBA-mine in Zambia: seam A (6.5% w/w FeS_2) and seam B (15% w/w FeS_2). The mean diameter of the coal-particles was around 50 μm.

Shake flasks experiments: Two types of shake flasks were used: cylindrical serum bottles (250 ml), sealed with rubber stoppers or flat-bottomed round flasks (250 ml), sealed with air-permeable plugs (Silicosen; Shin-Etsu Polymer Co., Ltd, Japan). The volume of the liquid phase was 50 ml or 100 ml, respectively. The flasks were inoculated (2% v/v) with a preculture, grown on standard medium with 0.2% FeS_2 and 0.01% YE. The flasks were incubated in reciprocal shaking (130 rpm) machines at 45°C.

Fermentor experiments: An 1.5 L lab-scale fermentor (Applikon, the Netherlands) was used. The working volume was 1.1 L. Air or a gas-mixture, consisting of 95% (v/v) air and 5% (v/v) CO_2 , were supplied at a flow rate of 10 L/h. The contents of the fermentor were mechanically stirred at 600 rpm and the temperature was maintained at 45°C.

Measurement of cell-numbers: Culture samples were taken and, after the settlement of the pyrite particles (5 min), the number of cells in the suspension was counted using a Burker-Turk counting-chamber. In each sample at least 300 bacteria were counted in order to obtain a reliable measurement of the cell-number.

Organic carbon measurement: The organic carbon contents of culture and supernatant (15.000 g; 3 min) were determined by injecting 20 μl in a Total Organic Carbon Analyser (Beckman), and converting the signal to ppm C with calibration curves obtained with potassium-phthalate. The difference between culture and supernatant was attributed to bacteria and expressed in ppm C.

Measurement of Fe^{2+} and Fe_{tot}: Samples were centrifuged at 15.000 x g for 3 min. Analyses for dissolved Fe-ions were performed with the supernatant fraction. Ferrous iron, ferric iron and total soluble iron were determined with the ortho-phenanthroline method.

Measurement of sulfate: After the removal of soluble iron, samples were analysed for sulfate by means of HPLC, using an anion-exchange chromatography column.

RESULTS AND DISCUSSION

Introductory remarks. The isolated bacterium had a broad temperature optimum from 40-50°C. At lower or higher temperatures the rate of pyrite oxidation was substantially reduced. Therefore, all the experiments were carried out at 45°C. The moderately thermophilic organism was able to oxidize pyrite between pH 1.0-2.0, with an optimum at about pH 1.5. All the experiments were started at pH 1.5.

Furthermore, it was found that an insignificant percentage of the total number of bacteria was attached to the pyrite-particles during leaching experiments with pure pyrite. For that reason bacterial growth could be followed by measuring the amount of biomass present in suspension.

Chemolithoheterotrophic growth on pyrite and yeast-extract. The isolate was able to grow heterotrophically on yeast-extract. In Fig. 1 it is shown that about 0.7×10^8 and 1.0×10^8 cells/ml were formed in media, containing 0.01 and 0.02% yeast-extract (YE), respectively. The addition of 0.2% FeS_2 to these media resulted in the formation of an increased number of bacteria. About 0.7×10^8 and 2.3×10^8 cells/ml were produced in media, containing 0.2% FeS_2 and either 0.01 or 0.02% YE, respectively. Comparison of the latter two numbers reveals that the biomass concentration did not increase in proportion to the YE concentration, but only by a factor 1.4. Nevertheless, these results strongly indicate that the bacterium can also grow chemolithoheterotrophically. In other words, the oxidation of pyrite yields extra energy for the assimilation of a greater part of the organic carbon compounds present in YE. Furthermore, it is noteworthy that little or no growth occurred in the medium, containing only 0.2% FeS_2.

In Fig. 2 the results are shown of experiments in which still higher concentrations of YE were used. In this case the difference between the organic carbon content of the culture suspension and the corresponding supernatant was used as a measure of the bacterial biomass concentration and was expressed in ppm C (mg C/L). The maximum amount of biomass formed in the medium, containing 0.2% FeS_2 and 0.01% YE was about 22 mg C/L. At 0.05% YE the maximum biomass concentration amounted to about 39 mg C/L. This means that only 1.8 times more bacteria were produced for a 5-fold increase of the YE concentration. At 0.1% YE the biomass concentration amounted to about 50 mg C/L. However, at this percentage inhibition of the bacterial growth became obvious, as can be judged from the lag phase of 6 days. At 0.01, 0.05 and 0.1% YE the organic carbon content of the culture suspension decreased during the growth phase from about 40 to 30 ppm C, 200 to 90 ppm C and 400 to 240 ppm C, respectively (not shown). This observation suggests that a substantial part of YE was dissimilated to CO_2 . Therefore, at an initial percentage of 0.2% FeS_2, the non-linear increase of the biomass concentration with increasing YE concentrations (in the range from 0.01 to 0.05%) can be interpreted as follows. Apparently, the oxidation of FeS_2 does not yield energy enough to assimilate all consumable organic compounds in YE, and partly dissimilation of YE occurs. This deficiency of energy production can be elucidated by introducing the parameter $PTR_{(max)}$ (mol FeS_2 /L.h), which represents the maximal rate (capacity) of transfer of pyrite from solid to the liquid phase. $PTR_{(max)}$ is, most probably, linearly proportional to the surface area of pyrite and, therefore, its value is not constant, but will gradually decrease during leaching experiments. Now, the shortage of energy can be envisaged as a consequence of the fact that in media, containing 0.2% FeS_2 at the start, $PTR_{(max)}$ is not as high as would be needed for the assimilation of all the YE.

In order to obtain further experimental evidence in support of this interpretation, growth was measured in media, containing a constant amount of 0.01% YE and increasing percentages of FeS_2 . In Fig. 3 the number of cells per ml is given in time for experiments with 0.2, 0.4 and 0.6% FeS_2. It can be seen that at an initial percentage of 0.4% FeS_2 the bacteria grew at a higher growth rate and that, indeed, more bacteria were formed than at 0.2% FeS_2 . However, increasing the initial percentage from 0.4 to 0.6% FeS_2 did not result in any further enhancement of the growth rate or the maximum number of cells formed. So, above a certain percentage of pyrite (in between 0.2 and 0.4%) the maximum amount of biomass formed is limited by YE. Furthermore, after one week of incubation the number of cells decreased in the experiments with 0.4 and 0.6% FeS_2. It can be argued that by that time $PTR_{(max)}$ is lower than required to sustain all the bacteria produced in the preceding growth phase, and consequently the cells lose their viability and partly lyse. In this respect, it is striking that the biomass seems to be more stable if the preceding growth phase had ended due to limitation by FeS_2, instead of by YE (see Figs. 1, 2 and 3).

The kinetics of pyrite oxidation. It was found that, after the initial growth phase, the oxidation of pyrite apparently could be characterized by a first order constant (k in 1/h) in each individual experiment. Plots of the logarithm of the remaining percentage of FeS_2 against time resulted in straight lines, the slopes of which were used to calculate the values of k. These first order constants were measured in experiments carried out under a variety of culture conditions. Open shake flasks, closed serum bottles and a well-mixed and aerated fermentor were used (see Material and Methods section for more details). The media contained different amounts of pyrite, but a constant percentage of 0.01% YE. In Fig. 4 the calculated values of k are given as a function of the initial concentration of FeS_2. It appeared that k was not independent of the pyrite concentration, as would be expected for a first order reaction, but was, on the contrary, inversely proportional. This remarkable observation on the kinetics of pyrite oxidation strongly supports the idea that YE limits bacterial growth above a given percentage of FeS_2. When the volumetric rates of pyrite consumption were calculated for all the experiments at the moment the maximum amount of biomass was (presumably) formed, it appeared that they were, more or less similar (ranging from 0.15 to 0.40 mmol FeS_2 /L.h), provided that the initial concentration of pyrite had been greater than about 20 mM. The interpretation of these results can be facilitated by introducing the Pirt concept of microbial growth and substrate consumption. Pirt (1965) postulated that bacteria require energy for growth as well as energy for maintenance. Applying this concept to batch leaching experiments with FeS_2, the following equation for the volumetric, total rate of pyrite consumption (r_p) can be derived:

$$r_p = \frac{\mu \,.\, x}{Y_p^{true}} + m_p \,.\, x \text{ (mol FeS}_2\text{/L.h)} \qquad [1]$$

The parameter μ represents the specific growth rate (1/h), x, the biomass concentration (g /L):Y_p^{true}, the molar growth yield on pyrite, corrected for maintenance (g/mol FeS_2) and mp the specific rate of pyrite consumption for maintenance (mol FeS_2/g.h). Thus, with regard to Fig. 4 it seems as if the bacteria once formed at their maximum number (xmax) only oxidize FeS_2 in order to fulfil their need of energy for maintenance purposes (r_p = m_p).(x_{max}). Therefore, it must be possible to initiate further growth and to increase the maximum amount of biomass, by adding an extra dose of YE at the moment FeS_2 is not limiting (i.e., when r_p < PTR($_m$ax)). The latter prerequisite can be met by starting the experiment at a high concentration of FeS_2. In Fig. 5 the decrease of the remaining percentage of FeS_2 is shown. The addition of an extra dose of 0.01% YE at the moment about 65% of the initial 73 mM FeS_2 was still present, indeed resulted in a stimulation of the oxidation of pyrite in comparison to the control, which did not receive extra YE. A similar stimulation was observed when 0.02% YE, instead of 0.01%, was added at the start of the experiment (not shown).

Effect of CO_2-enriched air on pyrite oxidation. In Fig. 1 it was demonstrated that virtually no growth took place in a medium, containing only FeS_2. Nevertheless, pyrite was still being oxidized at a substantial rate (not shown). Besides, in Fig. 6 it demonstrated that in the absence of YE the rate of pyrite oxidation increased in proportion to the inoculum size. Because the biomass concentrations were presumably far lower than those in the presence of YE, the specific rate of pyrite consumption for maintenance (m_p) must be much higher in the absence of YE. This raised the question as to whether our strain, under these circumstances, maybe exhibited a sort of uncoupling between energy production and growth (Senez, 1962). Moreover, it was reported (see Introduction) that some moderately thermophilic isolates, which showed a chemolithoheterotrophical lifestyle closely resembling that of our own isolate, were able to grow autotrophically as well, when they were offered CO_2-enriched air. For these reasons, the effect of CO_2-enriched air on the oxidation of pyrite was studied in a well-mixed and aerated fermentor. The remaining percentage of FeS_2 was determined on the basis of both iron and sulfate analyses. In Fig. 7 the results of such an experiment are shown in which during the first 13 days air was supplied to the suspension, containing 1% FeS_2 and 0.01% YE at the start. A clear stimulation of the oxidation of pyrite was found, when after day 13 the air was replaced by CO_2-enriched air (5% CO_2). A plausible explanation of the observed stimulation might be that the present bacteria are able to produce extra biomass by growing autotrophically in consequence of the elevated dissolved CO_2 concentration and that this presumably short, second growth phase comes to an end due to pyrite limitation. In another experiment, 0.2% FeS_2, instead of 1%, was added to the fermentor. In this case (Fig. 8) no stimulation of the pyrite oxidation was observed upon the switch from air to CO_2-enriched air after 4 days. This observation is in agreement with the above mentioned phenomenon (see Fig. 3), that in a medium, containing 0.2% FeS_2 and 0.01% YE, at the moment the maximum amount of biomass was formed, $PTR_{(max)}$ was already lower than would be needed for the maintenance of all the bacteria. Thus, although the conditions in the fermentor are conducive to autotrophic growth after the switch, the bacteria cannot grow, because pyrite is limiting and all the energy gained from the oxidation of pyrite is spent for the maintenance of the present biomass. In yet another experiment with 1% FeS_2 the stimulation of the oxidation of pyrite by CO_2-enriched air was confirmed (Fig. 9; upper line). Moreover, when CO_2-enriched air was supplied to the fermentor from the beginning of the experiment (Fig 9; lower line), the oxidation of pyrite proceeded at the same rate as that observed in the other experiment after the switch to CO_2-enriched air. When after one week normal air was supplied, no change in the rate of FeS_2 oxidation was found, presumably, because autotrophic growth did not occur any longer at the moment air was supplied to the culture and only O_2 was needed for the oxidation of FeS_2 in order to obtain energy for maintenance.

PRELIMINARY CONCLUSIONS

All the data presented in this paper on the kinetics of growth and pyrite oxidation can be accommodated to a model, which can be considered as our current working hypothesis for the moment. It comprises the following conclusions and assumptions: 1) The Pirt concept can be appropriately applied to describe leaching experiments with pyrite-particles. 2) The isolated, moderately thermophilic bacterium is able to grow heterotrophically and chemolithoheterotrophically in the presence of air, and autotrophically in the presence of CO_2-enriched air. 3) Growth is, under any circumstance, only possible if the volumetric rate of pyrite consumption (r_p) is lower than the maximum, volumetric capacity of transfer of pyrite from solid to the liquid phase ($PTR_{(max)}$)). 4) After cessation of the chemolithoheterotrophic growth phase due to limitation by YE, two consecutive phases in the oxidation of pyrite can be distinguised: first, r_p remains constant and is equal to the rate of pyrite consumption for maintenance and then, r_p decreases in a first order manner, when $PTR_{(max)}$ becomes lower than the r_p-value attained in the first phase.

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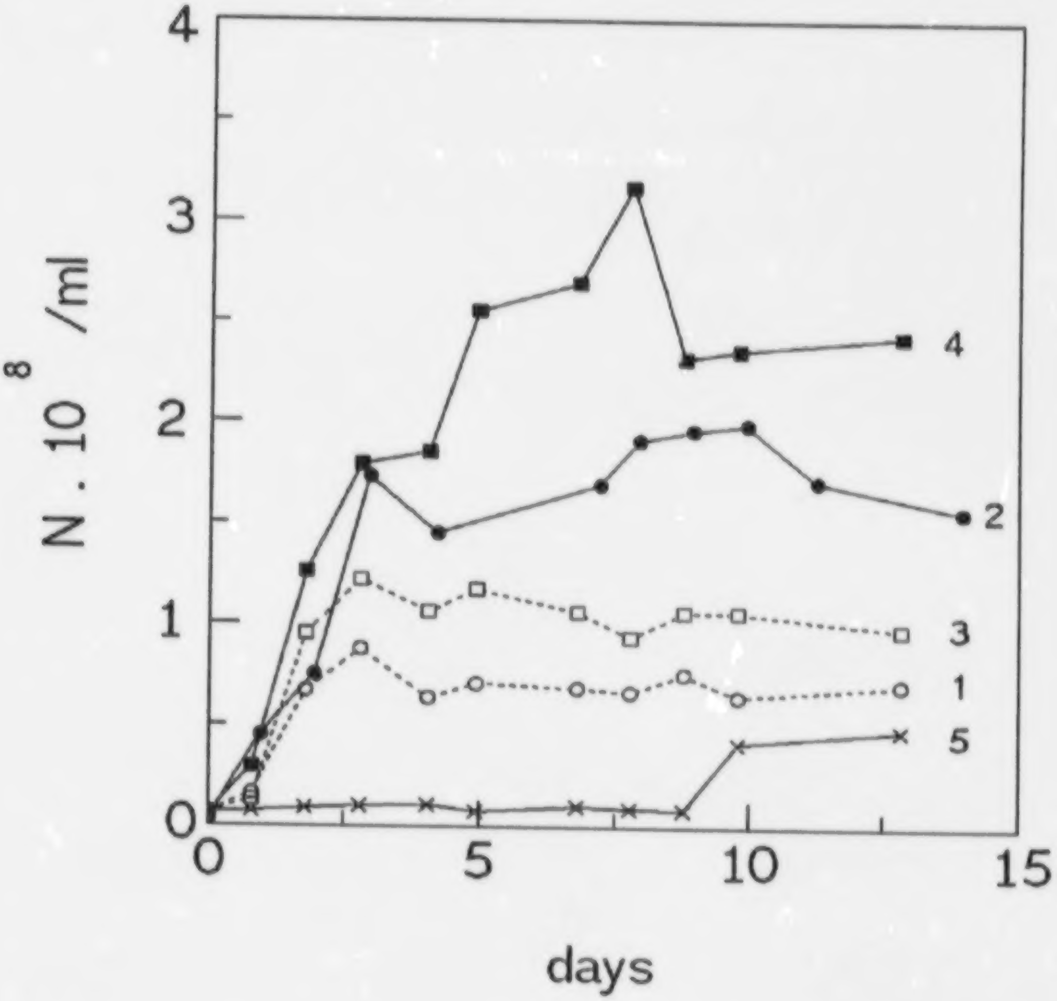

Fig. 1 Chemolithoheterotrophic growth on pyrite and yeast-extract.

The number of cell per ml versus time is given for growth in five shake flasks, containing standard medium supplemented with:

1) 0.01% YE (○)
2) 0.01% YE and 0.2% FeS_2 (●)
5) 0.2% FeS_2 (x)
3) 0.02% YE (□)
4) 0.02% YE and 0.2% FeS_2 (■)

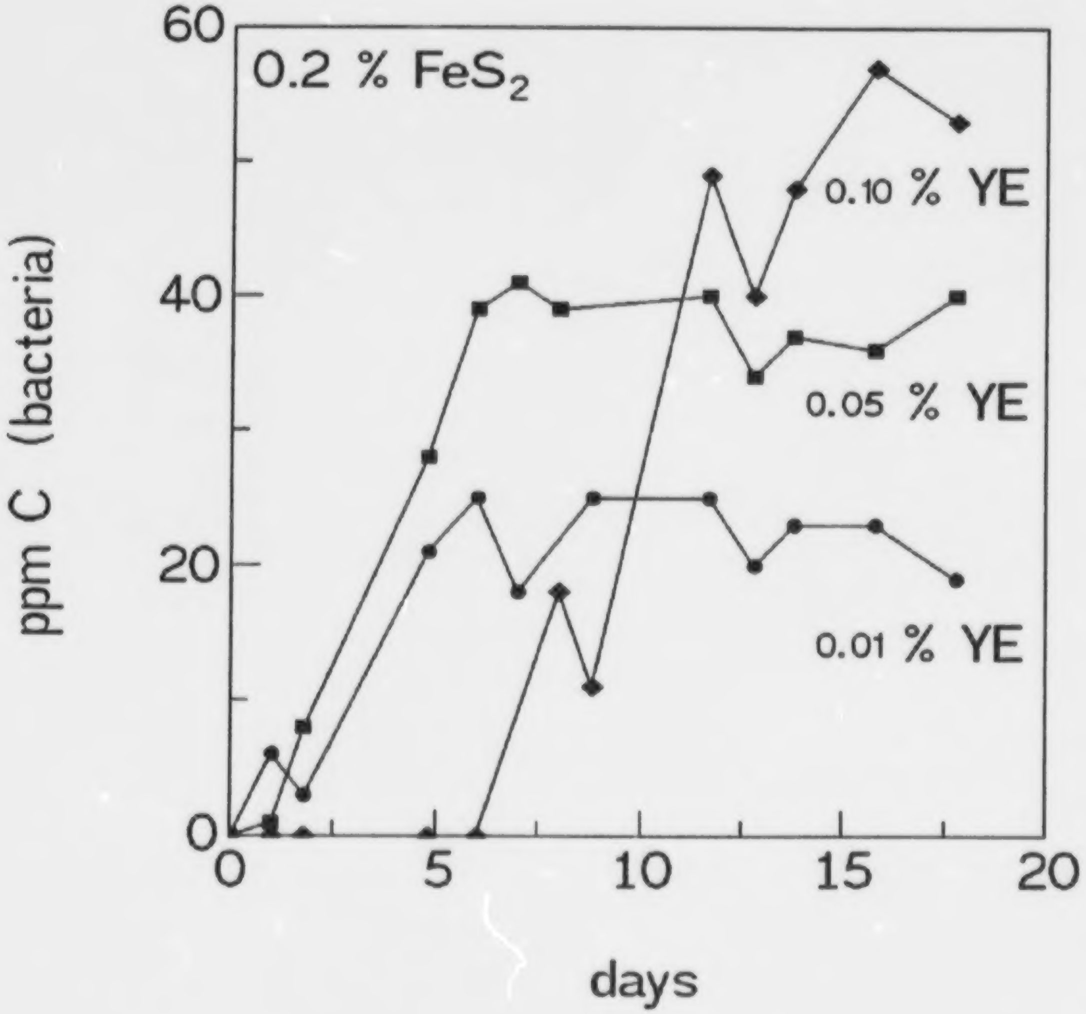

Fig. 2 Growth on 0.2% pyrite and variable amounts of yeast-extract.

The biomass concentration, given in ppm C, versus time is shown for growth in three serum bottles, containing 0.2% FeS$_2$ in standard medium, supplemented with 0.01% YE (●), 0.05% YE (■) or 0.10% YE (◆).

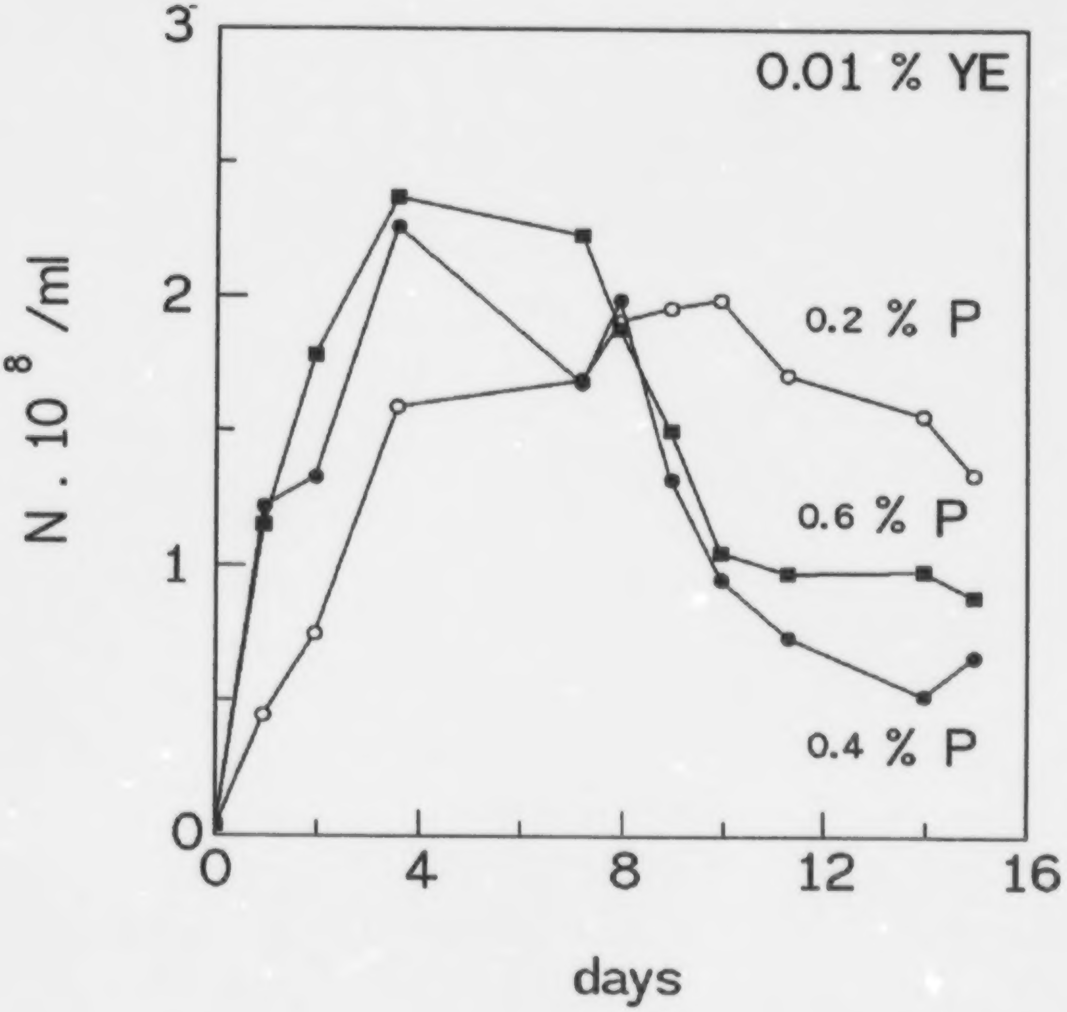

Fig. 3 Growth on 0.01% yeast-extract and variable amounts of pyrite.

The number of cells per ml versus time is given for growth in three shake flasks, containing 0.01% YE in standard medium, supplemented with 0.2% FeS_2 (○), 0.4% FeS_2 (●) or 0.6% FeS_2 (■).

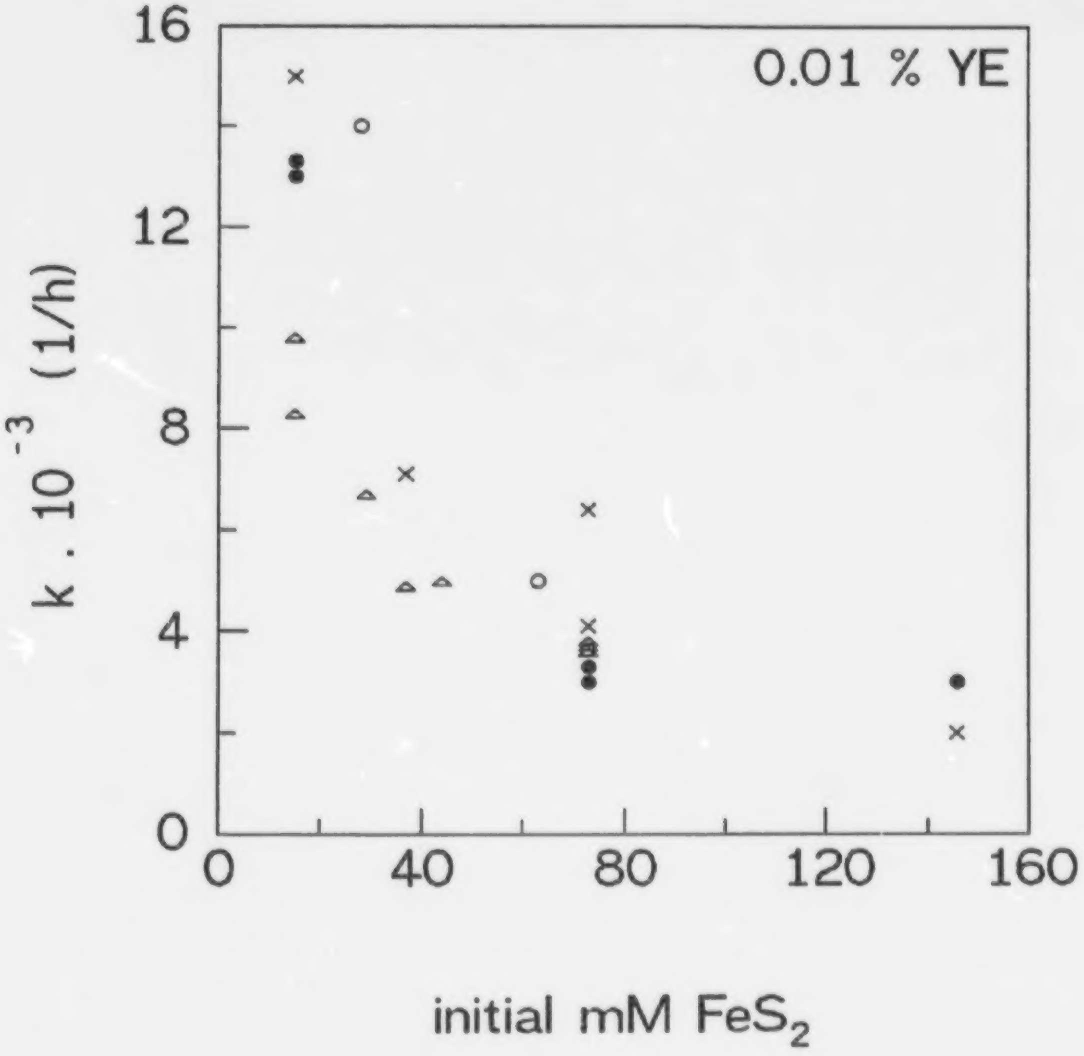

Fig. 4 First-order kinetics for the oxidation of pyrite? The first order constants are plotted against the initial concentration of pyrite. Values of k were calculated from experiments carried out with: shake flasks/pyrite (Δ, serum bottles/pyrite (x), shake flasks/coal (O) or fermentors/pyrite (●). In all experiments 0.01% YE was present at the start.

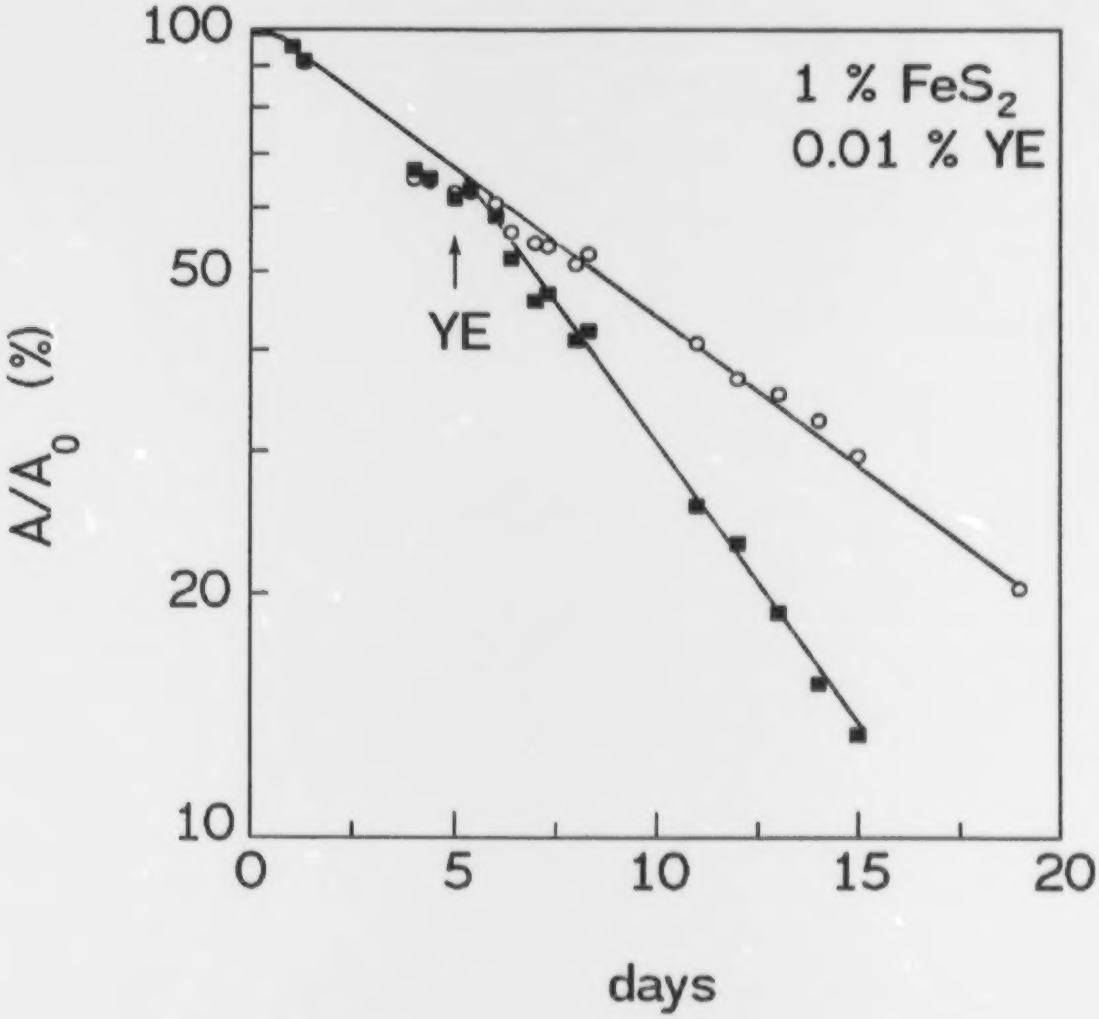

Fig. 5 Stimulation of pyrite (1%) oxidation by extra yeast-extract.

The remaining percentage of pyrite, given on a logarithmic scale, versus time is shown for two shake flasks (○,■), containing both 1% FeS_2 and 0.01% YE. To one flask (■) 0.01% extra YE was added after 5 days.

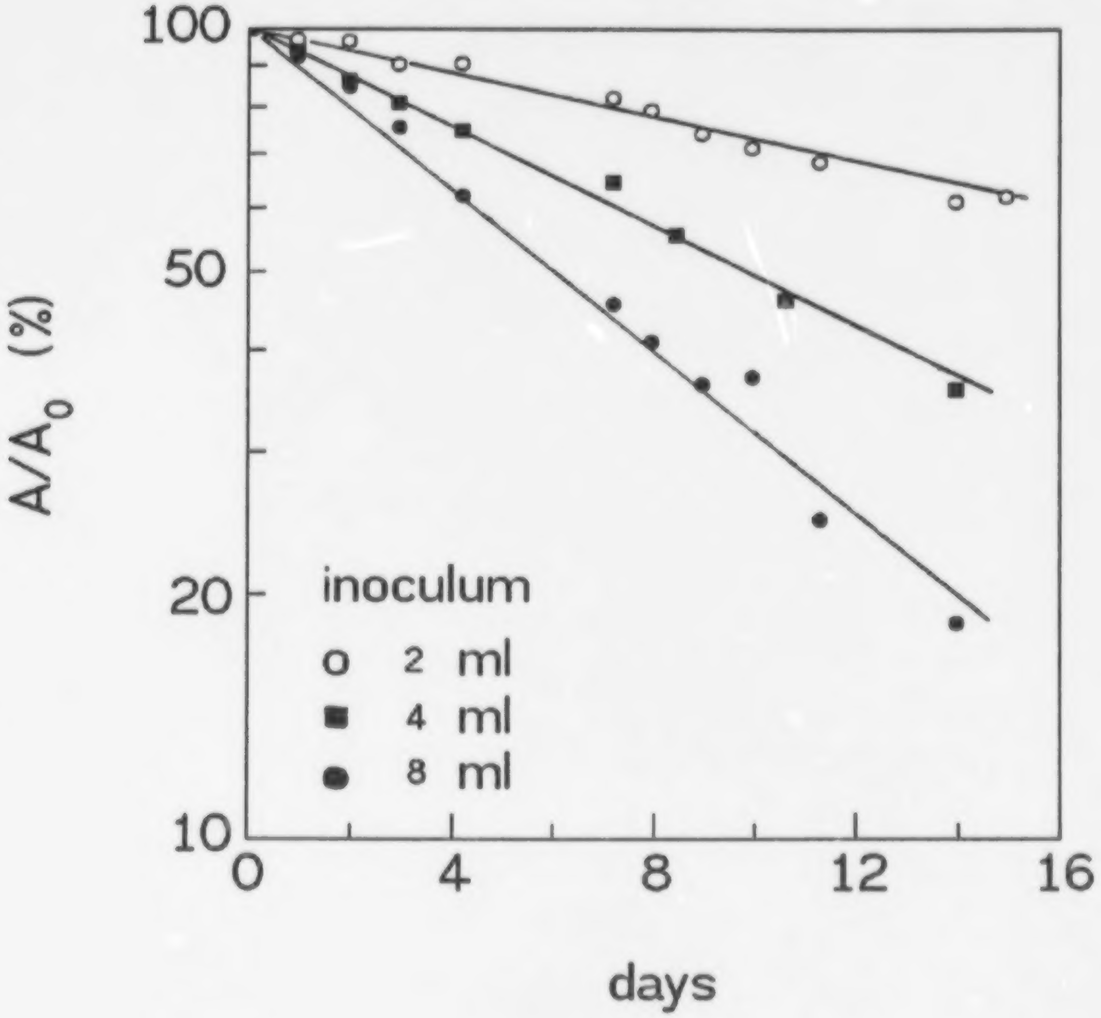

Fig. 6 Pyrite oxidation in the absence of yeast-extract.

The remaining percentage of pyrite (logarithmic scale) versus time is given for three shake flasks, containing 0.2% FeS_2 . The inoculum size (v/v) was 2% (○), 4% (■) or 8% (●).

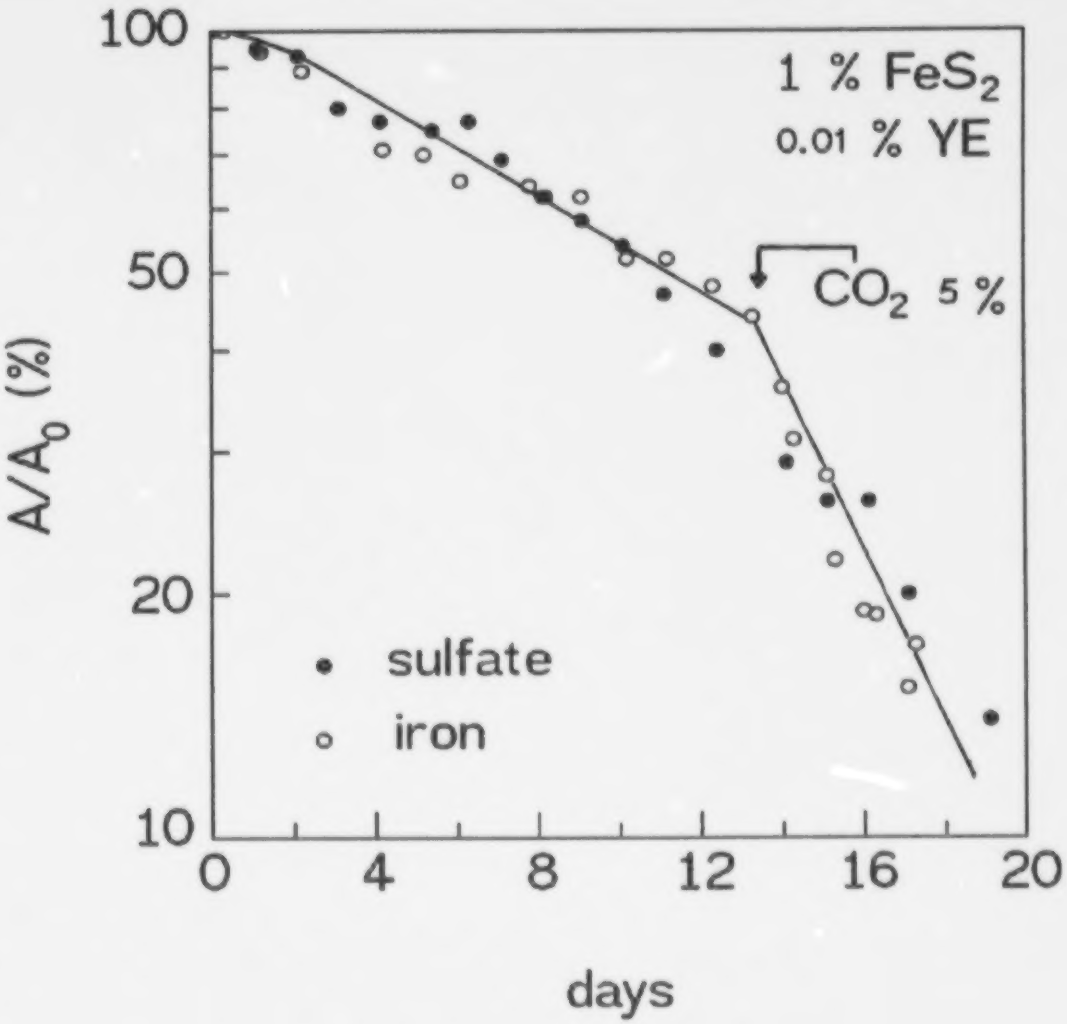

Fig. 7 Stimulation of pyrite (1%) oxidation by CO$_2$-enriched air.

The remaining percentage of pyrite (logarithmic scale) versus time is shown for a fermentor, containing 1% FeS$_2$ and 0.01% YE at the start. After 13 days the gas-supply was changed from air into CO$_2$-enriched air (5% CO$_2$). Values of A/A were calculated on the basis of sulfate (●) or iron (○) analyses.

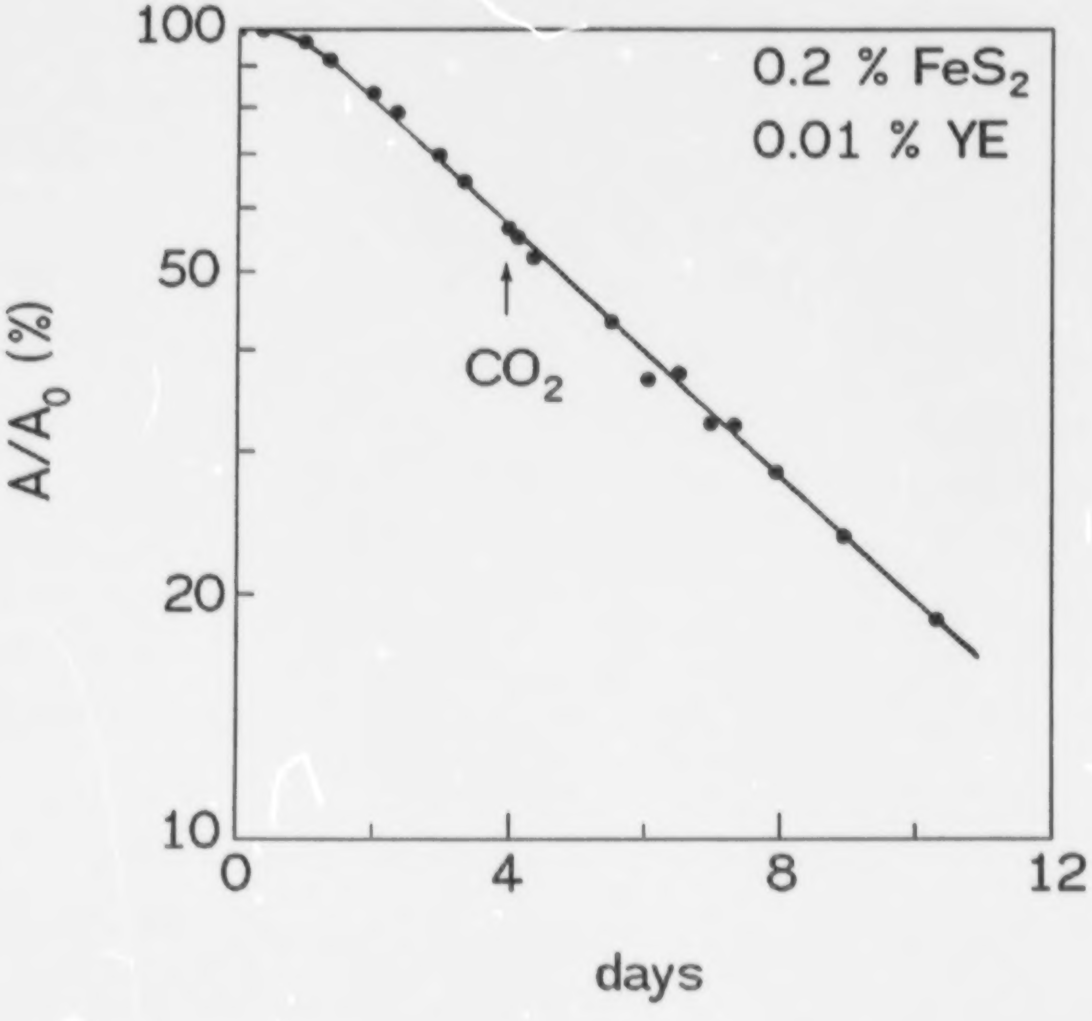

Fig. 8 No stimulation of pyrite (0.2%) oxidation by CO_2-enriched air.

The remaining percentage of pyrite (logarithmic scale) versus time is given for a fermentor, containing 0.2% FeS_2 and 0.01% YE at the start. After 4 days the gas-supply was changed from air into CO_2-enriched air (5% CO_2), Values of A/A were calculated on the basis of iron analyses.

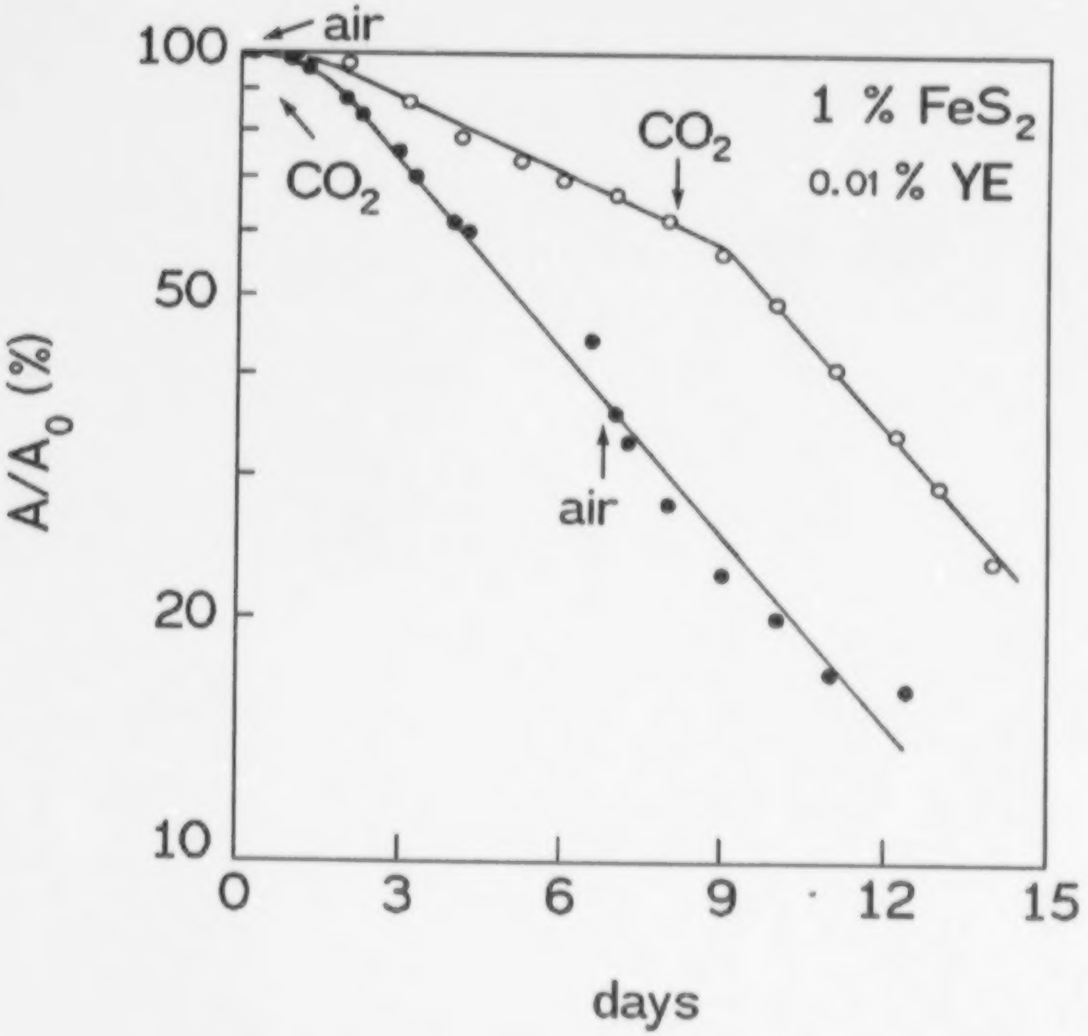

Fig. 9 Effect of a switch from CO$_2$-enriched air to air on pyrite oxidation. The remaining percentage of pyrite (logarithmic scale) versus time is shown for two fermentors, both contaning 1% FeS$_2$ and 0.01% YE at the start. One fermentor was supplied with air during the first 8 days, after which CO$_2$-enriched air (5% CO$_2$) was connected (○). The other fermentor received CO$_2$-enriched air (5% CO$_2$) during the first 7 days, whereupon air was supplied (●). Values of A/A were calculated on the basis of iron analyses (○,●).

FACTORS DETERMINING THE ECONOMIC FEASIBILITY OF REPROCESSING COAL BENEFICIATION WASTES.

S.B. Bhagwat, L.A. Khan, J.W. Baxter

Illinois State Geological Survey, Champaign

ABSTRACT

Reprocessing of coal beneficiation wastes can be profitable if carefully planned. The feasibility of reprocessing is affected by the quality and quantity of material available and the appropriateness of available technology but most of all by the ability to secure and meet a steady demand. The coal price is a crucial factor. Prices have not changed much over the past few years. The stability of prices at current levels is a positive factor. An important avenue could be opened in the future if cooperation between operators and state agencies for the reclamation of abandoned mine sites could be brought about. Major potential benefits may be realized by wide spread reprocessing of coal beneficiation wastes both economically as well as environmentally.

FACTEURS PERMETTANT D'ÉTABLIR LA FAISABILITÉ À BONS COÛTS DU RECYCLAGE DES DÉCHETS D'ENRICHISSEMENT DU CHARBON

S.B. Bhagwat, L.A. Khan et J. W. Baxter*

Illinois State Geological Champaign, IL 61820 E.-U.

RÉSUMÉ

Le recyclage des déchets d'enrichissement du charbon peut être rentable si il est bien planifié. La faisabilité du recyclage est affectée par la qualité et la quantité de matériel disponible ainsi que de l'appropriété de la technique disponible mais plus que tout par la capacité de sécuriser et de répondre à une demande constante. Le prix du charbon est le facteur crucial. Les prix n'ont pas beaucoup changé au cours des dernières années. La stabilité des prix aux niveaux actuels est un facteur positif. Une avenue importante pourrait être ouverte dans le futur si la coopération entre les opérateurs et les agences d'états concernant la réclamation des sites miniers abandonnés serait entreprise. Des profits potentiellement importants autant du point de vue économique qu'environnemental, pourraient être réalisés par l'étendue du recyclage des déchets d'enrichissement du charbon.

INTRODUCTION

Major coal producing states in the eastern United States, such as Kentucky, West Virginia, Illinois and others, generate mine gob, beneficiation gob and slurry in large quantities. Mine gobs result mainly from underground operations and are insignificant in states which mine coal primarily from surface mines. As a result Montana and Wyoming generate insignificant quantities of mine gob. Mine gob generally contains 2 to 4 percent coal and is economically unattractive for reprocessing. Beneficiation gob consists of large sized, non-combustible material which may contain up to 10 percent coal, although higher coal contents are occasionally encountered depending upon beneficiation technology used (1). However, much of the coal adheres to the rocks discarded in the process and makes reprocessing difficult. The elimination of mine and beneficiation gobs leaves only the slurries as possible candidates for reprocessing. Slurries contain varying amounts of coal depending upon how elaborate the beneficiation process has been. A survey of the 35 operating plants in Illinois indicated that the coal contents of slurries can be as high as 70 percent and may average 40 to 55 percent (2). Depending upon the mining technology used and the beneficiation practices followed, the coal content of slurries may average higher or lower than in Illinois. However, the trend to higher mechanization in mining is likely to generate greater amounts of fine material and therefore coal contents in slurries under current coal cleaning practices.

The significance of reprocessing of waste slurries in any coal producing state also depends on how much of the coal undergoes beneficiation. Low sulfur coals produced in some of the western states is marketed without beneficiation while over 90 percent of coal mined in Illinois is mechanically cleaned before marketing. Other eastern states average about 60 percent. In general, the high sulfur states or regions within these states are more likely to employ beneficiation. Illinois, for example, generates about 14 million tons of beneficiation gob and 6 million tons of slurries annually. About 3 million tones of fine coal is estimated to be lost in slurries yearly in Illinois alone. Together with those of other states, these losses represent not only an economic loss but also an environmental problem which cannot be ignored. Slurries from coal cleaning plants are normally deposited in ponds which permanently occupy large tracts of land and acids leached from the weathered pyrite in coal may be introduced into surface and ground water. Reprocessing the slurries from coal beneficiation plants thus offers economic and environmental opportunities that must be carefully and selectively seized to the benefit of the industry and the society as a whole. In the remaining part of this paper we present an analysis of factors that influence the feasibility or reprocessing coal beneficiation wastes. The factors will be divided into three categories related to the material characteristics, the technology of reprocessing and the economic environment.

NATURE OF MATERIAL

Having eliminated mine and beneficiation gobs from possible reprocessing, the slurry material from the beneficiation plants can be reprocessed before being deposited in slurry ponds. Additionally, or alternatively, material from slurry ponds can be extracted and processed. The characteristics of the material in either case will be different because of difference in settling patterns in ponds by particle size as well as by specific gravity.

The most important characteristic of the materials contained in slurries is its particle size distribution. To begin with, most material in slurries is smaller than a quarter inch in diameter. In Illinois, the -75 μm fraction is dominant with about 45% of slurry material in this size category (Fig. 1). The coal in slurries in Illinois is somewhat coarser than the rest of the material as a comparison of figure 2 with figure 1 would show. The beneficiation processes that generated these slurries were not suited to separate coal from non-combustibles in the fine size fractions involved. Any technology for reprocessing must, therefore, be able to accomplish this separation.

When slurries have been deposited into ponds, the natural difference in sedimentation of coal and non-combustibles can lead to some preconcentration of coal in certain areas of the ponds. Such preconcentrations can be made use of to reduce reprocessing costs. On the other hand, points of slurry discharge into the pond are often shifted over time, leading to several areas of preconcentration. As a result, selective extraction from areas of higher coal concentration becomes more difficult and expensive. Careful sampling of the entire pond can help identify areas of higher coal concentration, however, and help reduce costs.

Perhaps the most important single influence on the economic feasibility of reprocessing slurries is the coal content of the slurry because revenues from the operation are directly proportional to the coal quantity recovered. Low coal contents necessitate oversizing of equipment and thus higher investments. Larger plants also require greater operating and maintenance effort and more space which is often a major constraint, especially on existing coal beneficiation facilities.

Along with the coal quantity, the quality expressed by the Btu value of coal and its sulfur content determine the revenues. The Btu value is lowered if the coal has been exposed to weathering in ponds or if the ash content (i.e. non-combustibles content) of the recovered coal is high. Although the coal price is primarily determined by the Btu value, attention should be given to the sulfur content of coal. Conventional physical or physico-chemical can remove only the pyritic form of sulfur in coal. In the fine size fractions of coal slurries, pyrite and coal particles are difficult to separate. Attempts should be made to fine tune the cleaning process and eliminate as much pyrite as possible because lower sulfur coals enjoy a price premium for environmental reasons.

TECHNOLOGY OF REPROCESSING

The choice of technology can be crucial to economic success of secondary coal recovery operations. Basically, the technology for reprocessing has to be different from the one that created the waste slurry. As figure 3 shows, beneficiation plants in Illinois primarily depend on Jigs and Dense Medium equipment for coal cleaning. The choice of Jigs as the principal cleaning technology indicates that most plants do not consider processing of fines to be attractive. The reprocessing of slurries should, therefore, emphasize cyclones, concentrating tables, spirals and froth flotation as the technology of choice (3,4,5). In addition to being able to process finer material, this choice of technology minimizes the use of moving units and helps reduce costs. The only moving parts would be the pumps for the slurry, the shaking mechanism for the tables and the stirrers in flotation.

The material to be reprocessed can come directly as effluents of an existing coal beneficiation plant or be excavated from a slurry pond. In the latter case, some variation of dredging must be used and will increase the cost of coal recovery. The operator can influence the cost in a limited way by selecting the most cost effective dredging device after considering the purchase price, the repair and maintenance cost and the manpower needed for its operation.

The first step towards an economic operation consists of volume reduction. This is achieved by desliming the slurry i.e. removing the finest silt, usually -45 μm, and clay using diluted slurries in classifying cyclones. Investment in desliming reduces the equipment size in the rest of the plant and increases coal concentration because coal in slurries is coarser than the clay (see figures 1 and 2).

Further technological choice should depend on the particle size distribution of the deslimed material. Physical cleaning methods such as cyclones, spirals and tables should be preferred if the finer size fraction, 45 μm – 180 μm, is small. This size fraction can be better processed with physico-chemical methods such as froth flotation, but these are more sophisticated methods and are also more expensive. Experiments with slurries in Illinois indicate that either type of cleaning can handle the whole range of particle sizes occurring in slurries, although the efficiency of coal recovery may suffer if the equipment is not matched closely with the appropriate particle sizes. The correct composition of the process flowsheet must be determined in an opmization process that considers the following other factors:

* Availability of space, especially in an existing plant,

* Need for mobility of equipment depending upon total reserves available for reprocessing at a site,

* Coal recovery rates of equipment which can be influenced by the particle size and composition of the material,

* Overall cost per ton of recovered coal

Figure 4 shows a suggested flowsheet that uses physical cleaning methods for the +180 μm material and froth flotation for the -180 μm material after the desliming stage has eliminated the -45 μm fraction. Typically, where mobility is essential, concentrating tables should be avoided in favor of spirals. Spirals also save space in comparison with tables while offering the same convenience of being able to actually see the separation effect and fine tune the equipment.

Finally, consistency in product quality must be guaranteed. This is more difficult when material is being extracted from ponds and its coal content and particle size distribution fluctuate. The desliming stage serves to reduce quality fluctuations to a considerable extent. However, buffer tanks might be needed in some cases if the coal content in ponds varies frequently. Buffers add to the investments but help prevent oversizing of equipment otherwise.

ECONOMIC FACTORS

The most critical economic factor is the price of coal. The coal market has been soft for several years now and prices have remained stagnant. At a price of $20 per ton many reprocessing plants would be profitable in Illinois. A sensitivity analysis indicated that the minimum require coal content of the slurry would vary from 12 percent for an 80 t/hr processing plant to about 19 percent for a 40 t/hr plant. Thus, the importance of coal price is predictably linked with plant size and the coal content of the slurry.

The coal price also depends on the quality of coal, i.e. its Btu value, sulfur and moisture content and particle size. The last two also influence the transportability of coal. Dry, fine coal creates a dust problem during loading, transportation and unloading. It would be necessary to either use covered, and therefore expensive, rail cars or mix the fine coal with regular clean coal before transport. Other technological solutions such as agglomeration would be more expensive because the user would generally have to crush the coal again before burning.

If reprocessing plants are not part of an existing beneficiation facility with a marketing mechanism and major customers already in place, the market for reprocessed coal would be limited to small users such as institutions or small processing industries (6). This would require that low cost transportation for small daily quantities must be available. In most cases this would be local truck operators rather than railroads. In addition to matching the needs of a customer with the ability to deliver the needed quantities on a daily basis, the long term supply security must be guaranteed. For this, the reserve base in ponds should be adequate and/or primary beneficiation facility must exist.

When abandoned slurry ponds are reactivated for coal extraction, they may be treated as surface mine operations and be subject to reclamation requirements. Reclamation can be very expensive, costing several times the market value of land that may emerge as a result of reclamation. Recovery of coal from abandoned slurry ponds can reduce environmental problems and help reduce reclamation costs. In the absence of secondary recovery, federal funds are sometimes made available through state agencies for the purpose of reclaiming abandoned sites. It is, therefore, advisable to work closely with state authorities responsible for the reclamation of abandoned mine sites.

Apart from state involvement for environmental reasons as described above, the operations could be subject to interest rate fluctuations, the severity of which will depend on the borrowing policy. Higher levels of debt financing is to be expected with independent operators than with major companies in the coal business. Experience in Illinois indicates that operations for reprocessing of materials in slurry ponds are usually owned by independents who may need external financing. As a result of this and the need for investments in dredging equipment, slurry ponds must average 30 to 50 percent coal in the feed to be profitable as compared with 10 to 20 percent for reprocessing of effluents from beneficiation plants (2).

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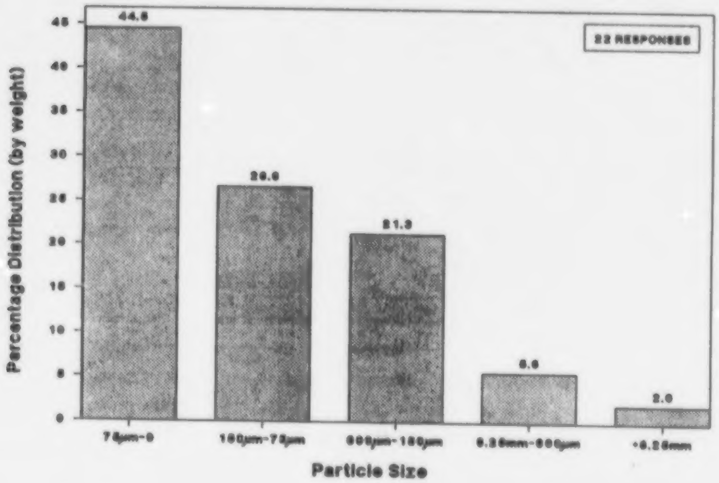

Fig. 1 Particle Size Distribution of the Material Rejected to the Slurry from Existing Plants

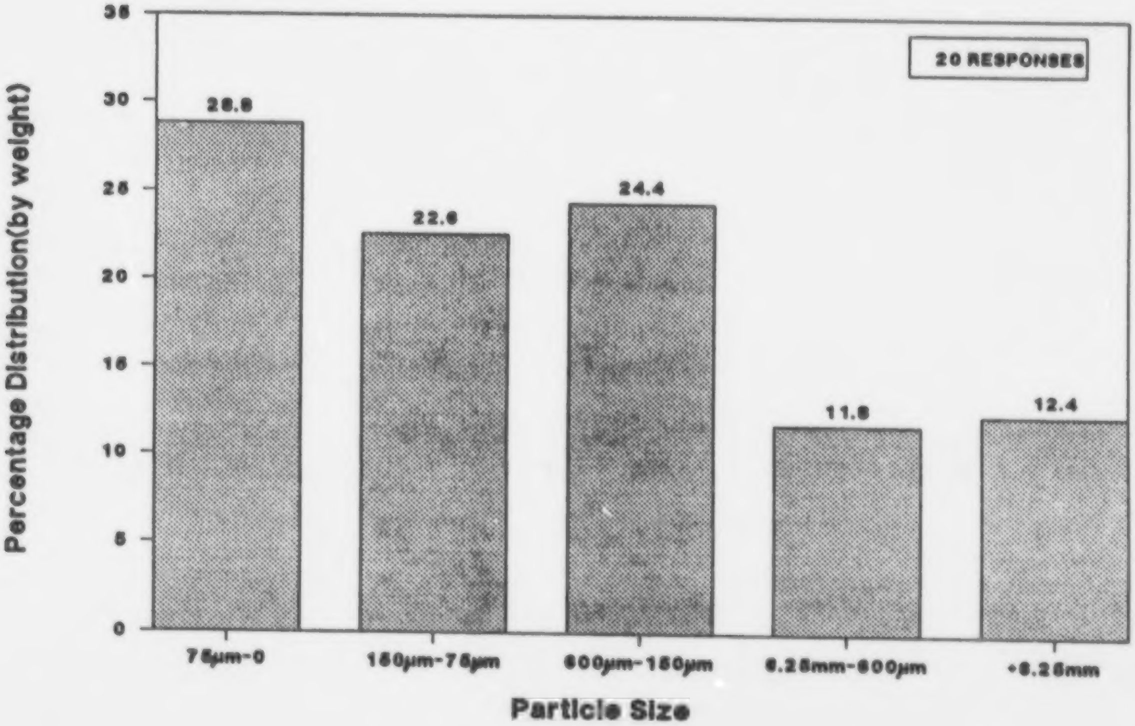

Fig. 2 Particle Size Distribution of the Coal in the Slurries of Existing Plants.

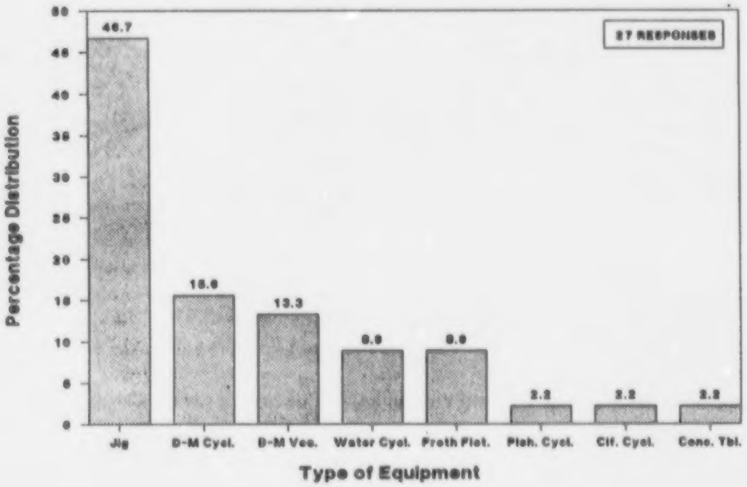

Fig. 3 Coal Cleaning Equipment Used by Existing Plants

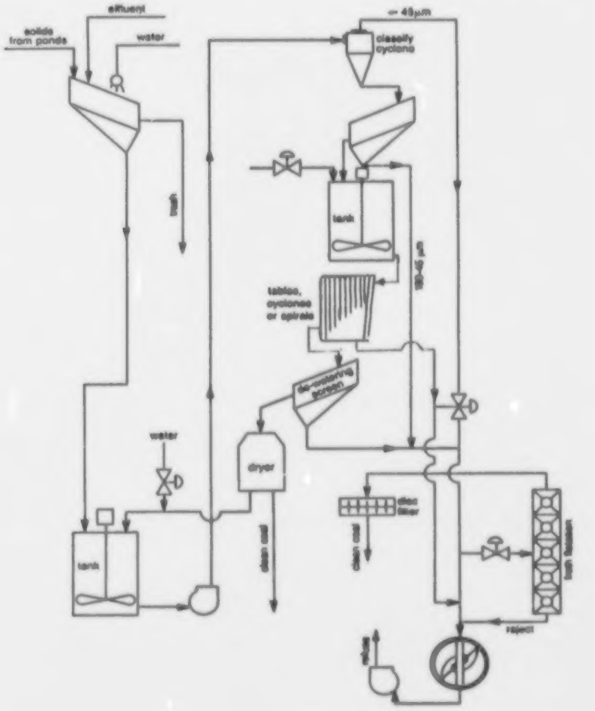
effluent
solids from ponds
water
trash
− 45μm
classify cyclone
tank
180-45 μm
tables, cyclones or spirals
de-watering screen
water
dryer
clean coal
tank
disc filter
clean coal
froth flotation
reject
refuse

Fig. 4 Flowsheet for +180 micrometer (physical) and 180 to 45 micrometer (flotation) particle size materials.

REMOVAL OF PYRITIC SULFUR FROM COAL BY *LEPTOSPIRILLUM* – LIKE BACTERIA

U. Merrettig, P. Wlotzka, U. Onken

*Universität Dortmund, FB Chemietechnik, Lehrstuhl für
Technische Chemie B, Postfach 500 500, D-4600 Dortmund 50,*

ABSTRACT

Microbial leaching processes with sulfur- and iron-oxidizing microorganisms can be used to remove pyritic sulfur from coal. For microbial leaching of sulfidic ores and coal, cultures of various *Thiobacillus* and *Sulfolbus* species have been used so far. The formation or insoluble precipitates containing ferric iron, which may occur during microbial leaching, can be avoided by operation at low pH. *Lestospirillum*-like bacteria, which were isolated at low pH during microbial leaching of pyritic flotation concentrate, were tested for oxidation of pyrite in coal. The microbial oxidation of pyrite was studied in a 4.5 l airlift fermenter at pH 1.5 and 100 g/L pulp density. By microbial leaching with *Leptospirillum*-like bacteria 85% of the pyrite was leached within 40 days. 30% of the leached pyrite sulfur was oxidized to elemental sulfur, and the other part was transformed to in soluble sulfate. Accumulation of elemental sulfur could be avoided by using a mixed culture of *Leptospirillum*-like bacteria and *Thiobacillus ferrooxidans*. Apart from oxidation of elemental sulfur neither the pure nor the mixed culture showed significant difference as to removal of pyrite.

EXTRACTION DU SOUFRE PYRITEUX DU CHARBON PAR DES BACTÉRIES DU GENRE *LEPTOSPIRILLUM*

U. Merrettig, P. Wlotzka, et U. Onken

Université de Dortmund, Technishe Chemie B, D-4600 Dortmund 50

RÉSUMÉ

Des procédés de lixiviation microbienne par des micro-organismes capables d'oxyder le soufre et le fer peuvent être utilisés pour extraire le soufre pyriteux du charbon. Jusqu'à présent, des cultures de différentes espèces de *Thiobacilli* et de *Sulfolbus* ont été utilisées pour la lixiviation microbienne du charbon et de minerais sulfurés. La formation de précipités insolubles contenant des ions ferriques, qui peut se produire pendant la lixiviation microbienne, peut être évitée par un fonctionnement à bas pH. Les bactéries du genre *Leptospirillum* isolées à bas pH pendant la lixiviation d'un concentré de flottation pyriteux, ont fait l'objet d'un essai en vue de l'oxydation de la pyrite dans le charbon. L'oxydation microbienne de la pyrite a été étudiée dans un fermenteur à ascension pneumatique de 4,5 L sous un pH de 1.5 et à une masse volumique de 100 g/L. Par lixiviation bactérienne avec des bactéries du genre *Leptospirillum*, 85% de la pyrite fut lessivée en moins de 40 jours. Il y a 30% du soufre pyriteux lessivé qui a été oxydé sous forme de soufre élémentaire, le reste fut transformé en sulfate soluble. L'accumulation de soufre élémentaire peut être évitée par l'utilisation d'une culture mixte composée de bactéries semblables au genre *Leptospirillum* et de *Thiobacillus ferrooxidans*. Sauf pour ce qui est de l'oxydation du soufre élémentaire, ni la culture pure ni la culture mixte n'ont présenté de différence significative quant à l'extraction de la pyrite.

INTRODUCTION

Sulfur contents of coal vary from traces to values as high as 10 or 12%. This sulfur leads to many problems when using coal for combustion or technical processes. Thus, it is necessary to find methods for reducing the sulfur content of coal.

Sulfur occurs in coal in different forms: organic sulfur which is bound to the coal matrix and inorganic sulfur compounds such as pyrite crystals (FeS_2) or small amounts of sulfate. Different chemical and physical methods have been used or proposed for removal of pyritic sulfur prior to combustion (Khoury 1981; Wheelock 1977), but most of these methods are unreliable because pyrite often occurs as small crystals within the coal matrix and thus is not easily available for reaction. Microbial leaching processes with sulfur- and iron-oxidizing microorganisms, which oxidize pyritic sulfur to soluble sulfate are better suited for the removal of this finely distributed pyrite from coal (Höne *et al.* 1987). Many publications about microbial coal desulfurization with different microorganisms like *Thiobacillus ferrooxidans* (Höne *et al.* 1987, Beyer 1987, *Sulfolbus* (Kargi and Cervoni 1983) and mixed cultures from *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* (Beyer *et al.* 1986; Dugan 1986) have appeared in the last few years.

The iron-oxidizing *Leptospirillum*-like bacteria were able to oxidize pyrite in pure culture or even in mixed culture with sulfur-oxidizing bacteria (Helle and Onken 1987). The produced ferric iron which acts as a leaching agent and chemically oxidizes pyrite to soluble ferrous sulfate and elemental sulfur. Ferric iron is reduced in turn to ferrous iron and is then regenerated by microorganisms (indirect leaching mechanism).

During microbial leaching of coal, formation of insoluble ferric iron precipitates may occur. This can be avoided by operation at low pH. *Leptospirillum*-like bacteria are able to continue dissolution of pyrite at pH values that inhibit *Thiobacillus ferrooxidans* (Norris 1983).

In this paper it is shown that *Leptospirillum*-like bacteria which were isolated during leaching experiments of a sulfidic flotation concentrate at low pH (Helle and Onken 1987) are also able to remove pyritic sulfur from coal.

METHODS

Microorganisms

Cultures used in this study were described earlier (Helle and Onken 1987). A culture containing *Leptospirillum*-like bacteria and a mixed culture containing *Leptospirillum*-like bacteria and *Thiobacillus ferrooxidans* were used. The cultures were maintained on a modified 9K medium (Silvermann and Lundgren 1959) containing 50 g/l pyrite concentrate as sole energy source. Pyrite concentrate was obtained from Preussag AG, Goslar (FRG).

Coal

Coal samples used in this study were obtained from Ruhrkohle AG, Bottrop (FRG). Particle sizes of 95% of the coal were found to be below 0.5 mm. Total sulfur content was 1.32% (w/w), pyritic sulfur 0.57% (w/w) and organic sulfur 0.74% (w/w). Elemental sulfur content and sulfate were less than 0.01% (w/w). Total iron content was 0.57%, pyritic iron 0.51%.

Experimental conditions

All leaching experiments were performed in a 4.5 L airlift fermenter with internal loop (Kiese *et al.* 1980) under non-sterile conditions. During all experiments the temperature was 30°C, aeration rate 8.8 L/min and pH 1.5. The reactor was filled with 4.2 L 9K medium (pH 1.5) and 430 g coal as sole energy source and inoculated with 100 mL bacteria culture. In the experiment without bacteria 100 mL

9K medium was used instead of the culture. For producing the inocula five shake flasks with 300 mL 9K medium (pH 1.5) containing 15 g pyritic concentrate were inoculated with 15 mL bacteria culture. After four weeks the flotation concentrate was removed from the bacteria by filtration and the culture was concentrated by centrifugation.

Analytic procedures

Total sulfur was determined by combustion (Schöninger 1961). The sulfate formed was determined by titration with barium chloride and thorin as indicator (Allenspacker 1982). Pyritic sulfur was analyzed by determination of pyritic iron. After extraction of the coal sample with hydrochloric acid pyrite was extracted with nitric acid.

Pyritic iron in the second extract, total iron and ferrous iron in the liquid phase were determined spectrophotometrically with phenanthroline (Deutsche Norm 1983). Elemental sulfur was extracted from coal with petroleum ether and determined in the extract by means of a spectrophotometric method (Bartlett and Skoog 1954).

Samples without further preparation were also analyzed for viable cells by the most probable number method.

Results and Discussion

In order to investigate the microbial removal of pyritic sulfur from coal three different batch fermentations were performed. Two different cultures were used as inocula: a pure culture of *Leptospirillum*-like bacteria and a mixed culture of *Leptospirillum*-like bacteria and *Thiobacillus ferrooxidans*. The third experiment was performed without bacteria as a control for chemical leaching. The sulfur balance for coal during the leaching experiments is shown in Figure 1. Total sulfur content of coal decreased by microbial leaching with *Leptospirillum*-like bacteria to 71% within an incubation period of 40 days (Figure 1 A). Only 62% of the total sulfur was found in the presence of the mixed culture after the same time. Without bacteria only 3% of the total sulfur was removed within 40 days. The observed decrease of total sulfur by microbial leaching is based on the oxidation of pyritic sulfur (Figure 1 B). The microbial removal of pyritic sulfur started after a lag-phase of 14 days, during which time the bacteria adapted to the coal. Then, the content of pyritic sulfur in coal decreased exponentially. Within 40 days 85% of the pyritic sulfur was removed by *Leptospirillum*-like bacteria and 83% by the mixed culture. The removal of pyritic sulfur by the two different cultures did not differ significantly. Therefore the presence of *Thiobacillus ferrooxidans* is apparently not essential for the oxidation of pyrite by the mixed culture. After 40 days the of pyrite oxidation became rather slow. Complete removal of pyritic sulfur was not possible because part of the pyrite crystals are enclosed within the coal matrix and can not be leached. During microbial oxidation of pyrite the *Leptospirillum*-like bacteria elemental sulfur accumulated; this effect was also observed during leaching without bacteria (Figure 1 C). Elemental sulfur content increased linearly up to 12% during chemical leaching. During microbial leaching by *Leptospirillum*-like bacteria a rapid increase of elemental sulfur was measured after 14 days which is congruent with the exponential decrease of pyritic sulfur. 30% of the removed pyrite was oxidized to elemental sulfur by both chemical and microbial leaching by *Leptospirillum*-like bacteria. The rest of pyrite was removed oxidized to sulfate.

The accumulation of elemental sulfur can be avoided by using a mixture culture of *Leptospirillum*-like bacteria and *Thiobacillus ferrooxidans*. After a slight increase within the first 14 days elemental sulfur content remained constant at 6%.

The increase in sulfate sulfur content in the liquid phase during the fermentations is shown in Figure 2. Chemical leaching causes a linear increase during the first 35 days and then a steeper increase by 1.4g/l after 40 days. Microbial leaching by *Leptospirillum*-like bacteria caused an increase of sulfate by 1.5 g/l after 40 days. The highest production of sulfate was measured in microbial leaching with the mixed culture. After 40 days the sulfate sulfur content increased by 3.3 g/l.

The results obtained from oxidation of pyritic sulfur content in coal are confirmed by measuring the total iron content in liquid phase. Results are shown in Figure 3. A linear increase in total iron content by 0.18 g/l was obtained by chemical leaching within 40 days. A rapid increase in total iron content was achieved in microbial leaching after a lag-phase of 14 days with both types of culture. After 40 days an increase in total iron content by 0.40 g/l was measured. The two different cultures also showed no significant difference in removal of pyritic iron.

The ferric iron content plays an important role in the indirect leaching mechanism. therefore it is interesting to know the ratio of ferric iron to total iron during chemical and microbial leaching of coal (Figure 4). In chemical leaching the amount of ferric iron slightly increased during the first 7 days and then remained constant at 30%. In microbial leaching the amount of ferric iron increased exponentially after 7 days. After 28 days all iron was ferric iron, with no significant difference found between the pure and the mixed culture. Because of this difference in ferric iron content between chemical and microbial leaching it is clear, that the ferric iron plays and important role as a leaching agent in the microbial removal of pyritic sulfur from coal. Ferric iron is responsible for the increase in elemental sulfur content in coal during microbial leaching with *Leptospirillum*-like bacteria. The accumulation of elemental sulfur may be avoided by using a mixed culture of *Leptospirillum-like bacteria and sulfur-oxidizing microorganisms.*

Viable cell counts of the different cultures during microbial leaching of coal are given in Table 1. In the mixed culture viable cell numbers indicated dominance of *Leptospirillum*-like bacteria. This result is in accord with prior studies which state that growth of *Thiobacillus ferrooxidans* is inhibited at pH values lower than 2.0 (Helle and Onken 1987). The number of viable cells of *Leptospirillum*-like bacteria in the mixed culture is only slightly lower than in the pure culture.

Our experiments show that *Leptospirillum*-like bacteria are able to oxidize pyrite in coal at low pH. This result is in agreement with the conclusions of others (Muyzer *et al.* 1987); these authors assume that the role of *Thiobacillus ferrooxidans* in microbial leaching is overestimated.

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Table 1

Viable Cell Number during Microbial Removal of Pyrite from Coal by Different Cultures

	Viable Cell Number (cells/ml)		
Time (d)	Leptosp.	Leptosp. and Thiob. ferroox.	
27	5,6 x 10^4		
40	4,7 x 10^4		
29		8,0 x 10^3	5,0 x 10^2
43		3,0 x 10^4	8,0 x 10^0

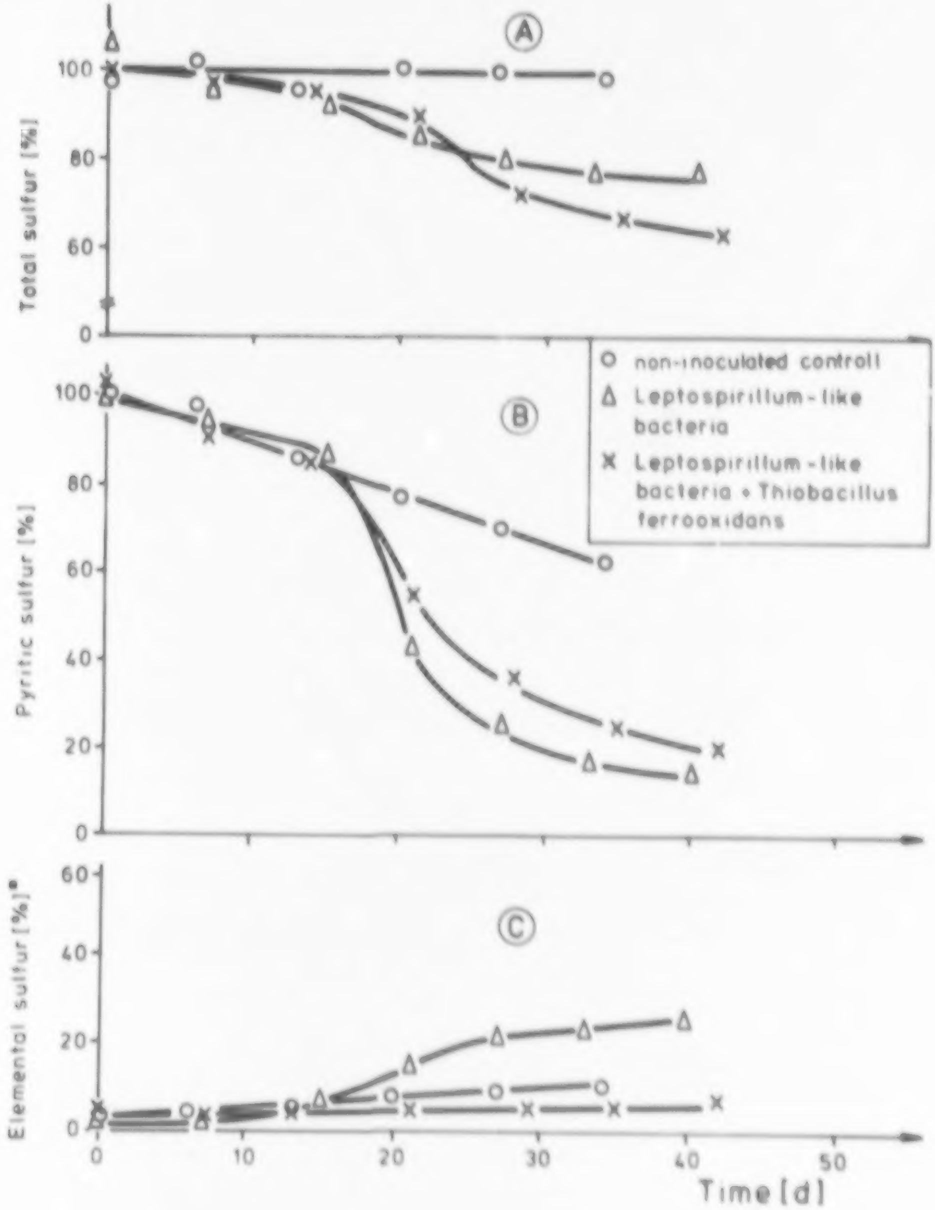

Fig. 1 Sulfur balance for coal during microbial leaching

A: total sulfur
B: pyritic sulfur
C: elemental sulfur
* Relative to the amount of pyritic sulfur;
100% means that all pyritic sulfur is oxidized to elemental sulfur.

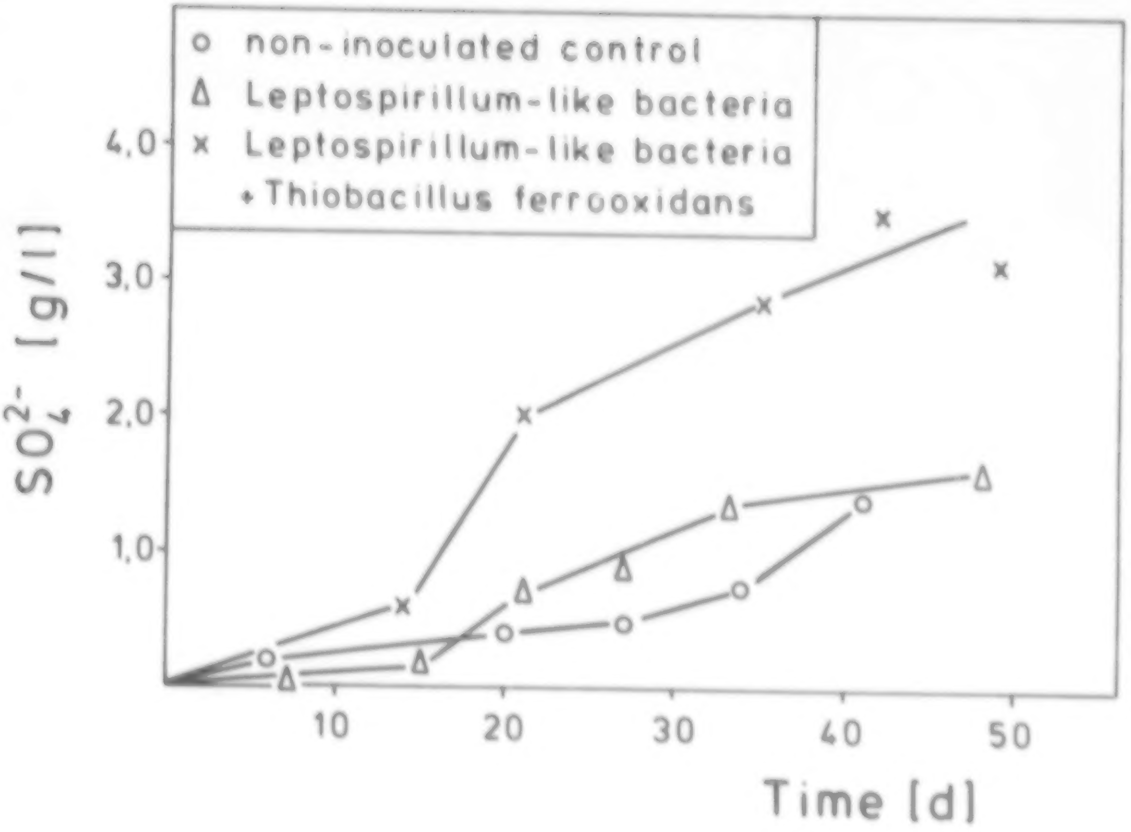

Fig. 2 Increase of sulfate sulfur in liquid phase

○ = non-inoculated control
▽ = *Leptospirillum*-like bacteria
X = *Leptospirillum*-like bacteria and *Thiobacillus ferrooxidans*

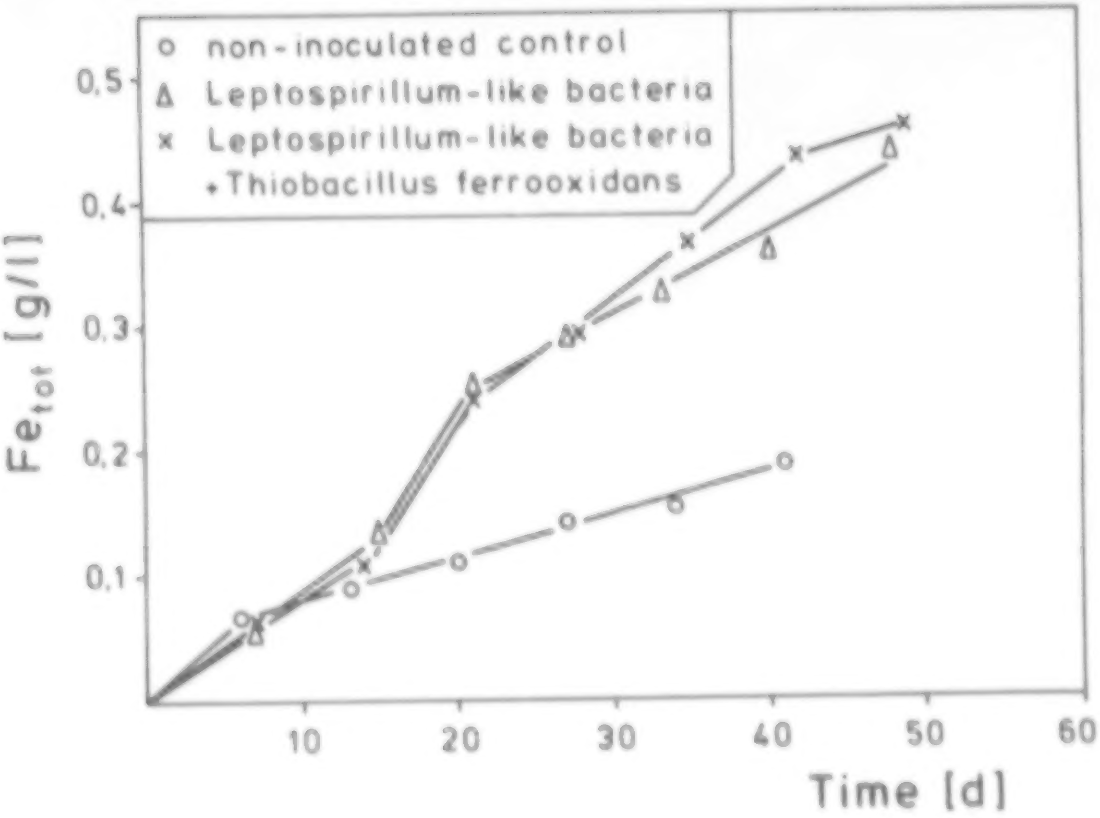

Fig. 3 Increase of total iron in liquid phase

○ = non-inoculated control
▽ = *Leptospirillum*-like bacteria
X = *Leptospirillum*-like bacteria and *Thiobacillus ferrooxidans*

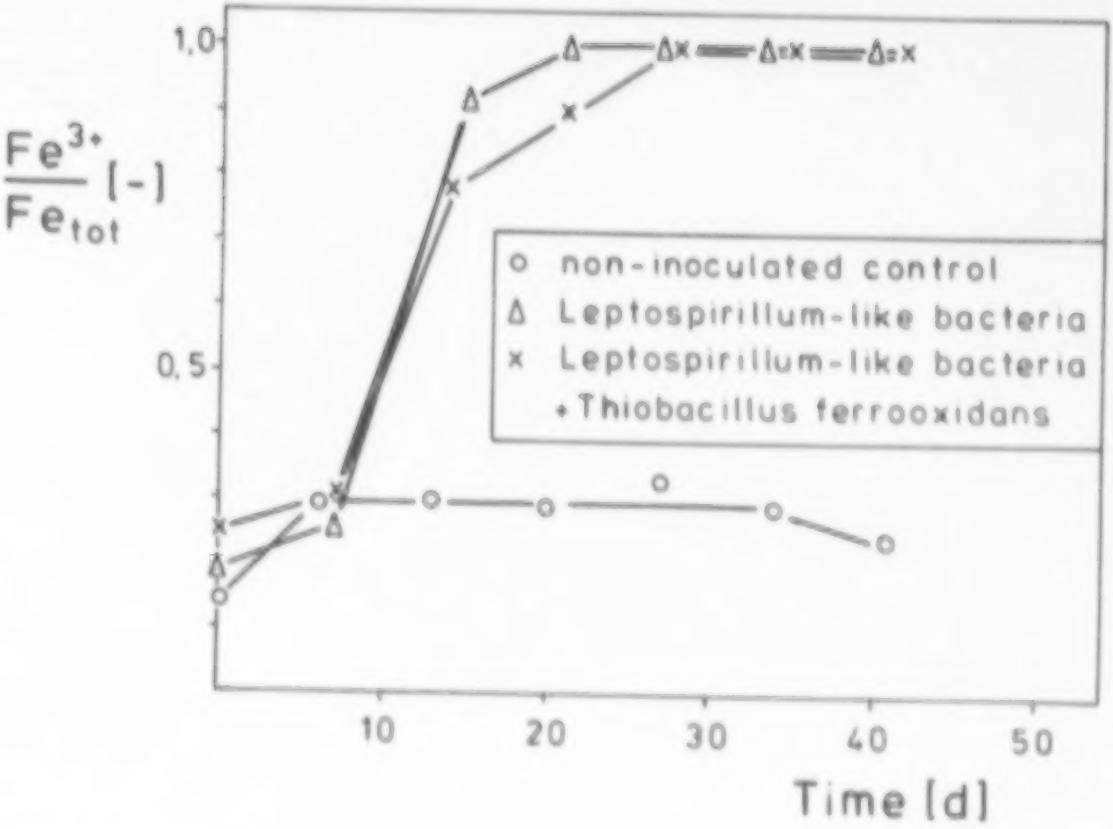

Fig. 4 Fractional amount of ferric iron in total amount of iron in liquid phase

O = non-inoculated control
∇ = *Leptospirillum*-like bacteria
X = *Leptospirillum*-like bacteria and *Thiobacillus ferrooxidans*